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PLASMIDS AND TRANSPOSONS

Environmental Effects and Maintenance Mechanisms

Edited by
COLIN STUTTARD
KENNETH R. ROZEE

PLASMIDS AND TRANSPOSONS

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Environmental Effects and Maintenance Mechanisms

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COLIN STUTTARD **KENNETH R. ROZEE**

Department of Microbiology
Dalhousie University
Halifax, Nova Scotia

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PREFACE

The meeting that provided the material for this book was organized as part of a continuing series of symposia sponsored by the Faculty of Medicine at Dalhousie University under the general title of ''The Scientific Basis of Medicine.'' This series brings together a relatively small number of active participants in a chosen health-related field to exchange data and ideas in the relaxed and congenial setting of Halifax, Nova Scotia.

In 1979, a rather broad view of public health was taken. We considered whether plasmids and transposons might be useful in providing a solution for pollution, and thereby to some extent offset the doctor's dilemma that mobile antibiotic resistance genes presently pose in the treatment of infectious disease.

Contributors to the symposium have given an appropriate evolutionary and ecological perspective to our topic, and note that genes that enhance host–cell adaptation to specific environments, whether by adding metabolic diversity or antibiotic resistance, are generally the most recent additions to the plasmid repertoire of an increasing range of bacteria. Also, several aspects of the evolution of composite conjugative plasmids through acquisition of transposons are considered from viewpoints that are both practical (the nullification of major therapeutic resources) and theoretical (the mechanisms of transposition and the maintenance and conjugational transfer of plasmids in bacteria).

Nosocomial infections are dealt with on several levels. Bacteria with multiple drug resistances are shown to be capable of taking up residence within the hospital environment and causing serious outbreaks of disease. The efficacy of acrylamide gel electrophoresis as a means by which clinical investigators may obtain evidence of similarity of pathogenic bacteria through plasmid identity is also covered. Participants also stressed in several ways the importance of plasmid analysis for the appropriate application of epidemiological control measures.

Manuscripts for this book were received from participants after the meeting and were edited to varying degrees in an attempt to enhance the uniformity of style. We apologize for any anguish which this may have caused some authors, but hope that a better book has resulted. We should like to express our thanks to all participants for their cooperation.

Colin Stuttard
Kenneth Rozee

Part I. ENVIRONMENTAL EFFECTS

GENETICS OF PLASMID-DETERMINED HYDROCARBON OXIDATION[1]

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INTRODUCTION

As components of the genome, plasmids help to determine the heritable phenotypes of particular bacterial strains. We can ask two related questions about the genetic role of plasmids: (i) a *functional* question about how plasmid genes control their transmission to daughter cells and determine novel cellular properties, and (ii) an *historical* question about the role that plasmids have played in the evolution of better adapted bacterial strains. The study of plasmid genes involved in hydrocarbon metabolism provides a perspective on these questions which is complementary to that which comes from work on antibiotic resistance, plasmid replication and plasmid transfer discussed elsewhere in this volume.

One point which deserves special emphasis is the need to examine plasmids and their genetic determinants in the context of particular host cells. Plasmid genes are not expressed in a vacuum. As Bryan points out in this volume, the ability of specific plasmids to increase resistance to antibiotics sometimes depends markedly on the particular host cell; and other

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[1] *This research has been supported by grants from the donors of the Petroleum Research Fund administered by the American Chemical Society, the National Science Foundation, and the Louis Block Fund of the University of Chicago. J. Shapiro was the recipient of a Public Health Service Research Career Development Award, and S. Benson and M. Fennewald were recipients of Public Health Service predoctoral traineeships.*

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papers have discussed the host dependence of plasmid-directed synthesis of phage receptors. We shall describe further examples of how the host cell can influence plasmid gene expression and also mention some cases where plasmid genes alter the expression of chromosomal genes. Thus, it is important to consider the plasmid-bearing cell as a complete genetic entity where several levels of interaction occur between the chromosome, plasmids and their respective gene products in determining cellular phenotypes.

MULTIPLE PLASMID-DETERMINED PHENOTYPES

The presence of a plasmid in a bacterial host can change its phenotype in a variety of ways. Surveying a large number of plasmids in different bacterial species (28) has led to the tentative identification of four basic groups of plasmid determinants.

1. *Determinants Involved in Plasmid Replication and Segregation to Daughter Cells*

These determinants are described in detail in the paper by Novick, and they control plasmid characteristics such as copy number per cell, incompatibility and host-range (i.e., the variety of species in which the plasmid can stably maintain itself).

2. *Conjugational Transfer Determinants*

These control plasmid transmissibility and associated characteristics of the plasmid-bearing strain, such as sensitivity to donor-specific phages.

3. *Determinants Which Regulate Interactions with Other Replicons and Extra-Chromosomal Elements*

These include plasmid sequences involved in chromosome mobilization and genes whose products inhibit the fertility of other conjugative plasmids in the same host, inhibit the growth of specific bacteriophages, or prevent the host from acting as a recipient in conjugational crosses (surface exclusion).

4. *Determinants Which Affect the Interaction of the Plasmid-Bearing Cell with the Environment*

These include genes for bacteriocin production and immunity, adhesion and pathogenicity factors, resistance to antibacterial agents (antibiotics, ions and radiation), and metabolism of environmental substrates (sugars, organic acids, aromatic and aliphatic hydrocarbons, detergents and pesticides).

Although there are exceptions, determinants in the first three groups appear to be very closely associated in many plasmids. Generally, all the members of one incompatibility group tend to share the same conjugational transfer determinants and show very similar fertility inhibition, phage inhibition and surface exclusion phenotypes. This suggests that groups of basic plasmid replicons acquired these various transfer and interaction determinants relatively early in their evolutionary histories. The last group of determinants, on the other hand, often shows a very different pattern of occurrence among plasmids isolated from nature because the same determinant can be found on plasmids of different incompatibility groups and plasmids within one group often have different "environmental" determinants. The best documented examples are the transposable drug resistance elements (see Foster and Kopecko, this volume), but there are also reports of transposable determinants for mercury resistance (30, and Friello and Chakrabarty, this volume), toxin production (29), lactose fermentation (10) and hydrocarbon degradation (21). It is therefore logical to conclude that genes which enhance the host cell's adaptation to very specific environments (growth on a particular carbon source, resistance to a particular antibacterial agent) are generally the most recent additions to the plasmid repertoire. These are apparently additions to the basic plasmid replicons which have evolved because of other selective pressures which we do not yet understand. Thus, the difference between a drug resistance plasmid and a degradative plasmid probably represents a very late branch in plasmid evolution rather than some fundamental division.

In our laboratory we have studied the IncP-2 plasmid group in *Pseudomonas*. Both the characterization of naturally occurring IncP-2 plasmids and laboratory recombination experiments supported the conclusions in the preceding paragraph. Members of this plasmid group all have a limited host-range, but they are the most common group of plasmids found in antibiotic resistant clinical isolates of *P. aeruginosa* (7,22, Jacoby, this volume). The camphor- and alkane-degradative plasmids CAM and OCT, originally isolated in *P. putida* strains (9,26) also belong to the IncP-2 group. Table I shows IncP-2 plasmids

TABLE I. Some Plasmids of the IncP-2 Group

	Plasmid	Phenotypic Determinants
Naturally Occurring	R931	Tra$^+$ Fi$^+$ (RP1) Fi$^-$ (FP2) Phi (B3 B39 E79 F116L G101 M6 PB1) Sm Tc Hg Uv
	R38	Tra$^+$ Fi$^+$ (RP1) Fi$^-$ (FP2) Phi (B3 B39 D3 E79 G101 M6 PB1) Sm Su Tc Hg Pma
	R130 (=pMG2)	Tra$^+$ Fi$^-$ (RP1, FP2) Phi (B3 B39 D3 E79 F116L G101 M6 PB1) Gm Sm Su Hg Uv
	Rms159	Tra$^+$ Fi$^+$ (RP1) Fi$^-$ (FP2) Phi (B3 B39 G101 M6 PB1) Cm Sm Tc Hg Pma
	R3108	Tra$^+$ Fi$^+$ (RP1) Fi$^-$ (FP2) Phi (B3 B39 D3 G101) Sm Su Tc Hg Pma
	pMG1	Tra$^+$ Fi$^-$ (RP1, FP2) Phi (B3 B39 D3 E79 G101 M6 PB1) Gm Sm Su Hg Uv
	pMG5	Tra$^+$ Fi$^+$ (RP1) Fi$^-$ (FP2) Phi (B3 B39 D3 E79 G101 M6 PB1) Ak Bt Km Su Tm Hg Pma
	RPL11	Tra$^+$ Fi$^-$ (RP1, FP2) Phi (B3) Cb Cm Gm Sm Su Tc Hg Pma
	CAM	Tra$^+$ Fi$^+$ (RP1) Fi (RP2) Phi (B3 B39 D3 E79 G101 M6 PB1) Cam Uv
	OCT	Tra$^-$ Alk
Laboratory Recombinants	CAM-R931	Tra$^+$ Sm Tc Cam Hg
	CAM-R3108	Tra$^+$ Sm Su Tc Hg Pma Cam
	CAM-pMG1	Tra$^+$ Gm Sm Su Hg Cam
	CAM-pMG2	Tra$^+$ Gm Sm Su Hg Cam
	CAM-OCT	Tra$^+$ Fi$^+$ (RP1) Fi (FP2) Phi (B3 B39 G101 PB1) Cam Alk Uv
	CAM-OCT-pMG2	Tra$^+$ Gm Sm Su Hg Cam Alk
	pMF581 (CAM-R931 x OCT)	Tra$^+$ Phi (B3 B39 D3 E79 F116L G101 PB1 M6) Sm Tc Cam Alk Hg Uv
	pMF582 (CAM-R931 x OCT)	Tra$^-$ Tc Alk
	pMF583 (CAM-R931 x OCT)	Tra$^+$ Sm Tc Alk

TABLE I (continued)

Plasmid	Phenotypic Determinants
pMF585 (CAM-R3108 x OCT)	Tra$^+$ Phi (B3 B39 D3 F116L G101) Sm Su Tc Hg Pna Uv$^-$ Cam Alk
pSB724 (R931 x OCT)	Tra$^-$ Tc Alk
pSB725 (R931 x OCT)	Tra$^+$ Tc Sm Alk Hg
pSB729 (R3108 x OCT)	Tra$^+$ Tc Sm Su Alk Hg

Data taken from references 12 and 22. The nomenclature for indicating conjugational transfer (Tra), fertility inhibition (Fi), phage inhibition (Phi) and other phenotypic determinants is given in reference 28.

which were characterized for a variety of phenotypic determinants, mainly in the laboratory of G. Jacoby. (Not all the plasmids were subjected to all tests.) The table includes naturally occurring plasmids and laboratory recombinants isolated by Jacoby and by us. These recombinants were readily found after selecting *P. aeruginosa* or *P. putida* clones which had maintained phenotypes determined by two distinct IncP-2 plasmids through two or three subclonings (12). In the first clone a majority of progeny cells had one or the other plasmid, but the few progeny which had markers from both plasmids generally showed stable inheritance of these markers on subsequent repurification and transfer. Thus, resistance and degradative markers were freely recombinable within plasmids of the IncP-2 group. In addition, Jacoby and Jacob showed that some of the drug resistance determinants from IncP-2 plasmids could insert into IncP-1 plasmid replicons in Rec$^-$ *P. aeruginosa* cells (20), but we were unable to find similar apparent transpositions of alkane degradation (*alk*) genes into IncP-1 plasmids. Not only did laboratory experiments show rearrangements of resistance and degradative markers, but the study of naturally occurring plasmids indicated that this recombination took place on a fairly constant background of genes for replication, transfer and replicon interaction. For example, most of the IncP-2 plasmids (with the exception of OCT) are conjugative and have similar fertility inhibition and phage inhibition patterns. The CAM and R38 plasmids showed identical behavior with the RP1 and FP2 plasmids and a number of *P aeruginosa* phages although their other determinants were very different. In contrast, the resistance and metabolic determinants of most naturally occurring IncP-2 plasmids varied markedly when they came from hosts isolated in different environments. Physical analysis of this plasmid group is complicated because all of its members are very large, generally well over 200 megadaltons in size (14,18). However, the limited restriction results to date indicate that both CAM and R3108 derivatives share many sequences although they are clearly different plasmids (14).

AROMATIC HYDROCARBON DEGRADATIVE PLASMIDS

A number of plasmids determine degradation of aromatic hydrocarbons and their oxidation products in *Pseudomonas* species. These include NAH, encoding enzymes for naphthalene degradation (11), SAL, encoding salicylate degradation (8), and TOL, encoding degradation of toluene and xylenes (32). These three plasmids all belong to the IncP-9 group which also includes drug resistance plasmids (Jacoby, this volume). Genetic experiments with these plasmids illustrate the similarity of degradative and resistance plasmids because it is easy to isolate hybrid resistance-degradative plasmids from SAL and TOL. We have shown that antibiotic resistance genes transpose into these plasmids (1,6), and other workers have shown that the degradative genes from TOL can be inserted into the RP4 IncP-1 resistance plasmid, apparently as part of a large transposable element (21,23).

TOL is the best-studied of the aromatic hydrocarbon degradative plasmids and provides a good illustration of host-plasmid interactions at several levels. The genes for oxidation of toluene to pyruvate and acetaldehyde can be transferred from *P. putida* to *E. coli*, either on an RP4-TOL hybrid plasmid or a TOL::Tn401 derivative, using antibiotic resistance as the selective marker in both cases (6,21,23). In the enteric host, expression of the degradative genes is very poor. *E. coli tol*$^+$ cells will not grow on toluene, xylenes or their oxidation intermediates, although some oxidation can be detected by the excretion of colored metabolites into the medium (21,23). Nakazawa *et al* assayed some *tol*-encoded enzyme activities in *E. coli* and found a low level of inducible expression (23). Thus, inducer enters the cell, but the *E. coli* transcription-translation apparatus works poorly on the *tol* DNA. Thus, at the level of protein synthesis, the nature of the host cell plays an important role in the expression of plasmid genes. It is important to keep in mind that there may be other levels at which the expression of plasmid genes can depend strongly on the host cells (such as transport of envelope proteins).

The pathway of toluene (xylene) oxidation was elucidated in *P. putida* strains carrying the TOL plasmid (Figure 1), and this pathway illustrates another aspect of plasmid-chromosome interaction. TOL actually encodes two successive pathways, one for oxidation of toluene to benzoate and another for oxidation of benzoate *via* catechol to pyruvate and acetaldehyde. The work of Worsey and Williams has shown that these two successive pathways are regulated differently because benzoate and its analogues will specifically induce the second one (33,34). Moreover, the SAL and NAH plasmids encode enzymes of very similar pathways for salicylate oxidation *via meta*-cleavage of catechol, and the main divergence between genes for NAH and

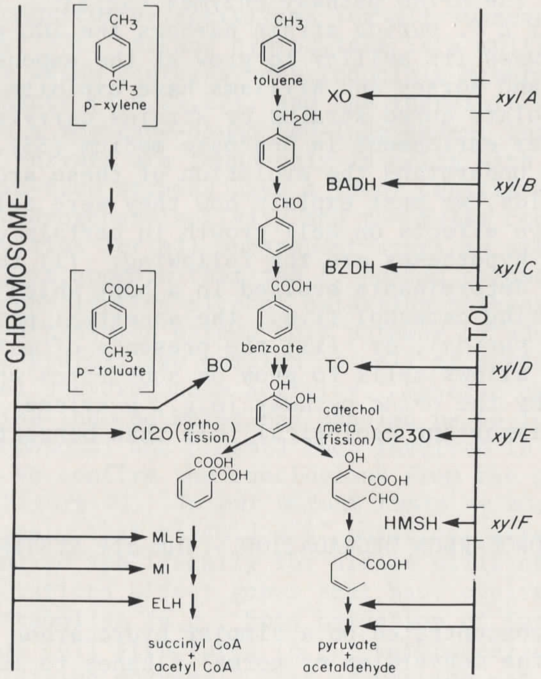

FIGURE 1. Oxidation pathway for toluene. The left-hand vertical line represents the P. putida *chromosome and the right-hand vertical line represents the TOL plasmid. The enzymes encoded by the chromosome are: BO=benzoate oxidase; C120=catechol 1,2-oxygenase; MLE=muconate lactonizing enzyme; MI=muconolactone isomerase; ELH=enol-lactone hydrolase. The enzymes encoded by the plasmid* xyl *loci are: XO=xylene oxidase; BADH=benzyl alcohol dehydrogenase; BZDH=benzaldehyde dehydrogenase; TO=toluate oxidase; C230=catechol 2,3-oxygenase; HMSH=2-hydroxymuconic semialdehyde hydrolase. C230, HMSH and subsequent enzymes are also encoded by the NAH and SAL plasmids. Adapted from references 31 and 32.*

TOL degradation lies in the initial pathway from hydrocarbon to organic acid (31). Thus, it appears likely that the toluene and naphthalene oxidation pathway genes evolved on IncP-9 plasmids from a common ancestor carrying genes for the *meta*-cleavage pathway. The presence of genes for the *meta* pathway for catechol oxidation on these plasmids is very interesting because they were isolated from nature in *P. putida* strains which carried an alternative catechol oxidation pathway (by *ortho*-cleavage) encoded by chromosomal genes (Figure 1; 32). This redundancy is even more paradoxical when we consider that the chromosomal *ortho* pathway is more efficient for the utilization of certain substrates, such as benzoate, than the plasmid *meta* pathway. But the presence of the plasmid prevents induction of the *ortho* pathway enzymes (32,33). In other words, the fact that a *P. putida* strain harbors the TOL plasmid actually *reduces* its ability to grow at the expense of certain substrates, and Worsey and Williams have exploited this phenomenon to isolate cured strains or strains carrying mutant plasmids after enrichment in benzoate medium (33). Clearly, if we are to understand the evolution of these aromatic hydrocarbon plasmids, we must explain how they were selected despite their negative effects on cell growth in certain environments. Two possible hypotheses are the following: (i) the plasmid *meta* pathway determinants evolved in a host which had no other way of oxidizing catechol (i.e., the ancestral plasmid did not evolve in *P. putida*), or (ii) the presence of *meta* pathway determinants allows cells to grow on substrates which cannot be oxidized by the *ortho* pathway (e.g., *p*-xylene) and which play a more important ecological role than benzoate and its precursors.

ALIPHATIC HYDROCARBON DEGRADATION - THE *alk* SYSTEM

We have concentrated on a simpler hydrocarbon oxidation pathway for the conversion of normal alkanes to TCA cycle intermediates ($R = CH_3(CH_2)_n$):

$$RCH_3 \xrightarrow[\text{hydroxylase}]{\text{alkane}} RCH_2OH \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} RCHO \xrightarrow[\text{dehydrogenase}]{\text{aldehyde}}$$

$$RCOOH \xrightarrow[\text{oxidation.}]{} \text{beta}$$

Different *Pseudomonas* strains carry out this pathway and grow on *n*-alkanes as sole sources of carbon and energy (24). Interestingly, the chain-length specificity of alkane utilization varies from strain to strain (24). For example, some strains will grow on alkanes as short as hexane and as long as heptadecane, whereas others will only grow on alkanes longer than dodecane, or longer than hexadecane, and so forth. In *P. aeruginosa*, this pathway for longer alkanes is probably catalyzed by chromosomally-encoded enzymes, because all isolates of this species which we have examined will grow on hexadecane. In contrast, *P. putida* and most strains of *P. aeruginosa* will not grow on *n*-alkanes shorter than undecane unless they carry an IncP-2 plasmid which contains the *alk*$^+$ genes originally found on the OCT plasmid.

Physiological experiments have shown that *alk*$^+$ *P. putida* strains have inducible activities for the first two steps of the alkane pathway (alkane hydroxylase and alcohol dehydrogenase) and constitutive activities for all subsequent steps of the pathway (17). Alkane hydroxylase is a multi-protein enzyme complex which contains both inducible membrane and soluble components (2,4,25,27), and the inducible alcohol dehydrogenase is a membrane-bound NAD-independent enzyme (5). Both of these enzymes are found only in cells with the *alk*$^+$ genes. Plasmid-free strains have a lower constitutive level of alcohol dehydrogenase activity and the fully constitutive level of activities for all subsequent steps in alkane oxidation. Thus, it appears that the *P. putida* chromosome encodes enzymes of a constitutive alcohol oxidation pathway and that the *alk*$^+$ IncP-2 plasmids extend this pathway by adding an inducible alkane hydroxylase complex as well as a redundant inducible alcohol dehydrogenase activity. Genetic analysis with nitrosoguanidine-induced mutations enabled us to identify a series of chromosomal and plasmid loci involved in alkane oxidation and so confirm the conclusions from the physiological experiments (Figure 2). In our mutant hunts we made one basic assumption, namely that plasmid *alk*$^-$ mutations affect genes which have evolved specifically for alkane utilization whereas chromosomal mutations affect genes that have evolved either for general metabolism (e.g., beta oxidation of fatty acids) or synthesis of cell structures (e.g., membranes) utilized by the alkane-oxidizing enzymes. This assumption was consistent with the presence of inducible activities only in plasmid-containing strains. Moreover, the chromosomal mutations we isolated fell into two classes: (i) those which blocked alcohol, aldehyde, and acid oxidation (*alcA*, *aldA*, *oic*, *ace*, *prp* mutations), and (ii) pleiotropic mutations which blocked expression of alkane hydroxylase activity in whole cells and altered other membrane-related properties of the plasmid-bearing strain (such as camphor oxidation, dye and antibiotic sensitivities; ref. 12). Thus, we felt justified in designating as *alk* genes only those plasmid loci which specifically control the alkane utilization phenotype.

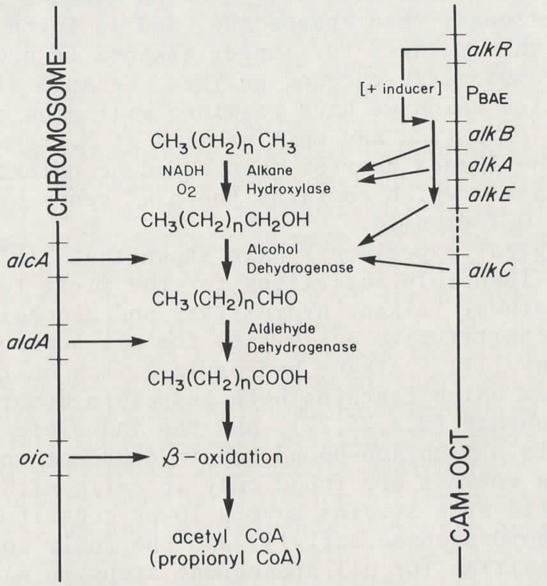

FIGURE 2. Oxidation pathway for n-*alkanes. The left-hand vertical line represents the* P. putida *chromosome and the right-hand vertical line represents IncP-2 plasmid DNA. The genes and gene products are explained in the text. Adapted from reference 13.*

The *alkA* and *alkB* loci determine, respectively, soluble and membrane components of alkane hydroxylase (2). The *alkC* gene product is required for synthesis of inducible membrane alcohol dehydrogenase (5,13). The *alkD* locus is defined so far by a single mutation and appears to encode a product which is required for synthesis of membrane alkane hydroxylase component and membrane alcohol dehydrogenase but *not* for synthesis of soluble alkane hydroxylase component (13). The *alkR* locus includes mutations which affect the regulation of both alkane hydroxylase components and of alcohol dehydrogenase, presumably because it encodes one or more positive regulatory proteins. There are alleles of *alkR* which specify non-inducible, constitutive and altered induction specificity phenotypes (13, 15).

Analysis of the organization of *alk* genes on IncP-2 replicons was complicated by the very large size of these plasmids. In order to do genetic mapping, we searched for transductional linkage between *alk*$^+$ and resistance or *cam*$^+$ markers on hybrid IncP-2 plasmids like pMF585 (Table 1). Unfortunately, we could find no linkage using the 38 megadalton (Mdal) F116L transducing phage in *P. aeruginosa* (19). So we decided to construct plasmids which had resistance markers linked to *alk* by using drug-resistance transposons. We found no insertions of the Tn*401* beta-lactamase transposon in or near the *alk* loci, even though we readily isolated *cam*::Tn*401* derivatives of CAM-OCT (1). However, the Tn*7* trimethoprim-resistance element gave us both *alk*::Tn*7* mutations and insertions readily co-transducible with various *alk* mutations (16). In order to isolate the linked insertions, we used a simple trick: we grew a transducing phage lysate on a random population of *P. aeruginosa* cells selected for the presence of CAM-OCT::Tn*7* plasmids and then transduced a *P. aeruginosa* (CAM-OCT *alk*) recipient to *alk*$^+$ Tp^r with this lysate. Such transductants would arise after infection with phage carrying the appropriate *alk*$^+$ segment and a nearby Tn*7* insert. (Incidentally, our failure with Tn*401* and success with Tn*7* illustrates the importance of trying several transposable elements when attempting *in vivo* genetic manipulations.) Figure 3 summarizes the results of measuring genetic linkage to two different Tn*7* inserts, which we have labelled Tn*7*$_{320}$ and Tn*7*$_{322}$. To our surprise, our *alk* point mutations were located in three separate clusters: *alkRD*, near Tn*7*$_{320}$ and far from Tn*7*$_{322}$; *alkBA*, near Tn*7*$_{322}$ and far from Tn*7*$_{320}$; and *alkC*, unlinked in F116L transduction to either of these two inserts or to *alkB, A, R* and *D* point mutations (13). Based on cotransduction frequencies, we estimated a distance of 42 kilobases between the *alkRD* and *alkBA* clusters (13). Thus, contrary to our initial expectations, the *alk* genes did not appear to occupy a small discrete portion of CAM-OCT DNA.

Analysis of the properties of *alk*::Tn*7* mutations caused by Tn*7* insertion into *alk* genes gave us additional information

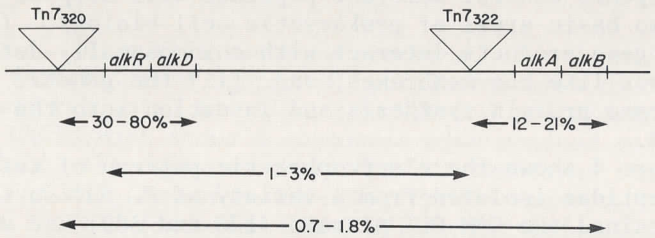

FIGURE 3. Genetic map of the alkRD *and* alkBA *clusters. The numbers inside the double-headed arrows indicate the range of cotransduction frequencies of markers in each cluster with either of the nearby Tn7 insertions. Adapted from reference 13.*

about the organization and control of these loci. Insertions into the *alkR* region gave pleiotropic mutants which were not inducible for either hydroxylase component or for alcohol dehydrogenase (13). This is precisely what one would expect for insertions into a gene encoding a positive regulator protein. Insertions into *alkC* behaved like point mutations of this locus and lead uniquely to loss of alcohol dehydrogenase activity. The characteristics of insertions into *alkB* and *alkA* revealed two new features of the *alk* system: (i) there was at least one additional alcohol dehydrogenase gene (called *alkE*) linked to *alkBA*, and (ii) *alkB, alkA* and *alkE* constituted an operon transcribed in the order *B - A - E*. These conclusions came from the observation that *alkB*::Tn*7* insertions were polar on the expression *alkA* and *alkE* (i.e., these insertions blocked synthesis of soluble hydroxylase component and alcohol dehydrogenase), that *alkA*::Tn*7* insertions were polar only on expression of *alkE* (i.e., blocked alcohol dehydrogenase synthesis), and that deletion of Tn*7* inserted into either *alkB* or *alkA* relieved that polar effect on *alkE* (i.e., on alcohol dehydrogenase expression) (13).

EFFECT OF alk^+ PLASMIDS ON THE *PSEUDOMONAS* CELL ENVELOPE

One of the interesting features of the alkane utilization system is that the substrate is hydrophobic. Consequently, we expected that its metabolism would involve the cell envelope to a much greater extent than the metabolism of soluble substrates such as sugars and amino acids. Hydrocarbon substrates will accumulate in the interior of bacterial membranes, and we know that the initial oxidizing activities for alkanes are associated with membranes. Therefore, we have investigated changes in *Pseudomonas* envelope proteins associated with regulation of the *alk* system. More detailed knowledge of how the *alk* genes control membrane peptides will help us understand two basic areas of prokaryotic cell biology: (i) how plasmid gene products interact with chromosomally-determined structures like the membranes, and (ii) the genetic control of membrane protein synthesis and insertion into the cell envelope.

Figure 4 shows the electrophoretic pattern of total membrane peptides isolated from a variety of *P. putida* strains. Two contained the CAM-OCT plasmid (123 and 380) and were grown under both inducing and non-inducing conditions. As expected, induction of the *alk* system resulted in the appearance of several new membrane peptides. On this gel, three are visible at positions corresponding to peptides of approximately

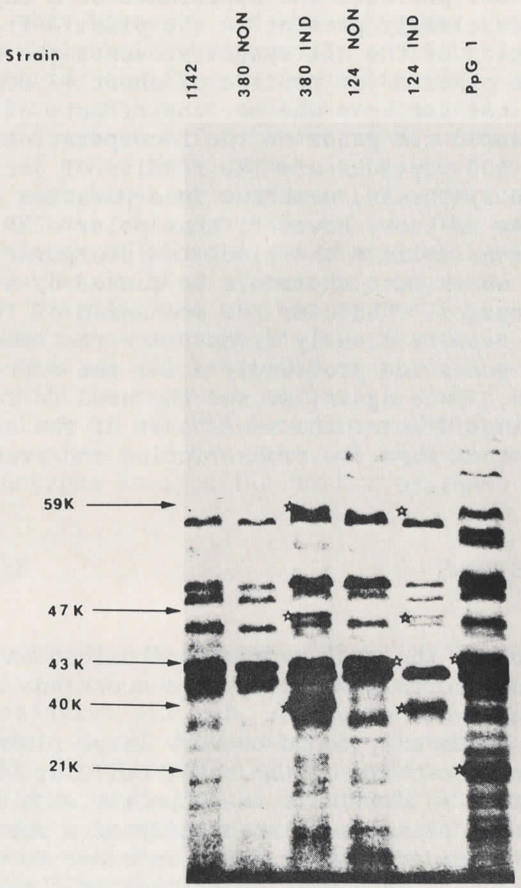

Figure 4. P. putida *membrane proteins. Total cell envelopes were isolated from various* P. putida *cultures by centrifugation and subjected to polyacrylamide-SDS gel electrophoresis. The photograph shows a coomassie blue stained gel. From left to right, the cultures are: PpS1142 (contains the CAM plasmid), PpS 380 (contains CAM-OCT), PpS385 induced for* alk *expression, PpS124 (contains CAM-OCT), PpS124 induced for* alk *expression, and PpG1 (contains no plasmid). The stars indicate the position of peptides whose appearance is controlled by plasmid determinants.*

product of the *alkE* gene (3). More interesting from the perspective of plasmid-host interactions are the effects of IncP-2 plasmids and the *alk* genes on the expression of chromosomally-encoded membrane peptides. The presence of either CAM (in strain 1142) or CAM-OCT prevents the appearance of a major 21,000 dalton peptide clearly present in the plasmid-free strain PpG1. Induction of the *alk* system prevents the appearance of at least one other major peptide of about 43,000 daltons (3). We do not yet know whether the effects of IncP-2 determinants and induced *alk* genes on the incorporation of these 21,000 and 43,000 peptides are the results of action at the level of protein synthesis, membrane incorporation or protein degradation. We do know, however, that polar *alkB*::Tn*7* and *alkA*::Tn*7* mutations relieve the block to incorporation of the 43,000 peptide, which must therefore be caused by a product of the *alkBAE* operon. Whatever the mechanism of these effects may be, our results clearly demonstrate that the expression of plasmid genes can profoundly alter the expression of chromosomal genes. Once again, we see the need to consider the expression of plasmid determinants as part of the activity of a total genome rather than the transcription and translation of an isolated template.

SOME UNANSWERED QUESTIONS

Our detailed look at the *alk*$^+$ hydrocarbon oxidation genes on IncP-2 plasmids has certainly raised more questions about their evolution than it has answered:

--Why are these genes only found on very large plasmids that constitute up to one-third of the total cellular DNA (14)? This observation is difficult to reconcile with the notion that degradative plasmids exist chiefly as a mobile reservoir of metabolic genetic information in order to minimize the total DNA content in a particular taxonomic group.

--Why aren't the *alk* genes clustered in a small region? If the sole objective in evolving a coordinated set of genetic determinants were to maximize their distribution among genomes, then we might expect them to be located in a restricted segment of DNA which could easily recombine or transpose onto a large number of different replicons.

--Have all the loci we identified as *alk* genes really evolved specifically for alkane (or related hydrocarbon) degradation? It may be that the *alk* genes evolved only on IncP-2 plasmids because their expression requires plasmid gene products which originally arose because of their importance in the production of other phenotypes.

--Are there silent *alk* genes on Alk$^-$ IncP-2 plasmids, such as CAM and the R factors? The dispersion of *alk* loci on CAM-OCT and the ease with which Alk$^+$ IncP-2 recombinants are formed suggest that perhaps not all three *alk* clusters are needed to change an Alk$^-$ plasmid to an Alk$^+$ plasmid.

--In what bacteria did the *alk* genes evolve? The plasmid-determined alcohol dehydrogenase activity is superfluous in wild *P. putida* and *P. aeruginosa* strains, and it is therefore difficult to see how it could have evolved in those species.

The *alk* system may turn out to be unique so that detailed analysis of other hydrocarbon degradative genes will not reveal the same kinds of complexities. However, we do not believe that this will prove to be so. The questions raised by the redundancy of plasmid and chromosomal pathways for catechol oxidation in strains carrying aromatic hydrocarbon degradative plasmids are similar to those posed above. Regardless of how idiosyncratic the *alk* system is, our experience tells us that an understanding of plasmid evolution will require a shift in research emphasis -- away from accumulating a longer list of plasmid-determined phenotypes and towards a detailed analysis of how selected plasmid genes control certain phenotypes in specific hosts.

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DISCUSSION

Bryan: Have you localised the protein band which disappears on introduction of CAM-OCT?

SHAPIRO: No, we haven't done that. The gels I showed are linear polyacrylamide gels and it's sometimes difficult to resolve some of the smaller peptides. We've now gone to a system of using gradient gels which enables you to see very many more bands and much more sharply defines them. You have to be careful, however; for example, if you want to see what happens when you introduce a plasmid and what peptides are made, you should try several different gel systems. Change the pH of your buffer, and the concentration of the gels, because in some gel systems some of these bands are piled up on top of other bands, but in *that* gel system they were resolved and in certain gradient gel systems they are resolved. We haven't done that exhaustively yet. We just started out with the three inducible peptides because we expected the proteins to be in the inner membrane where we knew the enzyme activity was located.

Clewell: Do you think any larger predictions can be made about the association of plasmid products and membranes?

SHAPIRO: Well I think that replication (and there's evidence for this in the case of F too), as well as conjugational transfer, definitely involve membrane proteins. I think that people have started to look at this and have started to detect plasmid gene products in membranes. I don't know how much work has been done in looking at chromosomal gene products and the effects that plasmids have on them. However, I think we need to have a more complete picture of effects, both in the cytosol as well as in the membranes. I think that much of the DNA which we now consider to be silent has some kind of regulatory role which may be subtle in that you can delete that DNA from a plasmid and it will still replicate in the laboratory, but it may have greater physiological significance out in the environment.

Vining: Are these plasmid-coded degradative pathways catabolite repressible? Are they under cyclic AMP control and could this have anything to do with the lack of expression in *E. coli*, for instance?

SHAPIRO: They are catabolite repressed, at least the alkane system is. Now I don't know if it's under cyclic AMP control. We did some not very serious experiments on that and didn't see much. Catabolite repression in *Pseudomonas* is somewhat different from that in *E. coli*. For example, substrates like succinate which are good non-repressing substrates in *E. coli* give the most repression in *Pseudomonas*. I think that in the Nakazawa experiments it's known that *Pseudomonas* RNA polymerase and *E. coli* RNA polymerase are somewhat different. Possibly it reflects differences in polymerase recognizing promoters. Some people have taken *Pseudomonas* genes and put them in *E. coli* and vice versa, as Irving Crawford is doing; and then you can get secondary mutations where specific genes are expressed better. If it turns out, for example, that those are promoter mutations then it would seem to indicate that the RNA polymerase is involved, and of course that's really part of catabolite repression.

PLASMIDS AND DISSIMILATION OF SYNTHETIC ENVIRONMENTAL POLLUTANTS[1]

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INTRODUCTION

Over the last several decades, a group of highly toxic hydrocarborn , *viz.*, man-made halogenated organics, have been released into the environment in massive amounts as pesticides or herbicides, refrigerants or propellants, fire retardants or simply as household or industrially useful compounds. Since naturally-occurring compounds with carbon-halogen bonds are rather rare, the presence of halogen atoms on such organic molecules renders such compounds refractory to microbial attack. This has resulted in significant bioaccumulation of many pesticides and other halogenated hydrocarbons in the environment. Such accumulation has in turn exerted strong selective pressure on the microorganisms for their rapid disimilation, as many of these compounds are extremely toxic to the microbiota. The purpose of this article is to discuss the evolution and role of plasmids that code for biodegradation of synthetic chlorinated compounds.

PLASMIDS AND EVOLUTION OF NEW METABOLIC FUNCTIONS

It is now well known that many hydrocarbon degradative

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[1] *The work was supported, in part, by a Genetic Biology grant (PCM79-17526) and an ASRA grant (PFR79-05499) from the National Science Foundation.*

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genes occur naturally in the form of plasmids in various *Pseudomonas* species (1). A list of such plasmids is given in Table 1. Because hydrocarbons such as xylene, toluene, n-octane, are toxic, the plasmid nature of the hydrocarbon degradative genes, by analogy with antibiotic resistance, is believed to have played a major role in the rapid spread of hydrocarbon degradative competence among the pseudomonads, in order to enable them to detoxify such hydrocarbons rapidly and effectively. It now appears that gene clusters specifying partial bioconversion of synthetic chlorinated hydrocarbons to acidic products that can perhaps enter the metabolic pool of other microorganisms are emerging in various soil and aquatic bacteria. Fisher, Appleton and Pemberton (23) have recently demonstrated the occurrence of a 58 megadalton (Md) transmissible plasmid pJP1 in *Alcaligenes paradoxus*, which specifies bioconversion of the pesticide 2,4-D to 2,4-dichlorophenol. The evolution of a conjugative 2,4-D plasmid appears to have played a major part in the lack of persistence of 2,4-D in the environment. The evolution of a similar type of plasmid encoding bioconversion of p-chlorobiphenyl (pCB) to acidic intermediates such as p-chlorobenzoic acid in enteric bacteria such as *Klebsiella pneumoniae* or *Serratia marcescens*, isolated from areas of the Hudson River sediments contaminated with PCB (polychlorinated biphenyl) has also been reported (24).

Table 1. List of Degradative Plasmids

Plasmid	*Degradative pathway*	*Transmissibility*[a]	*Size (x 10^6)*
CAM	Camphor	+	>100[b]
OCT	n-Octane	-	>100
SAL	Salicylate	+	55,48,42
NAH	Naphthalene	+	46
TOL	Xylene/Toluene	+	76
XYL-K	Xylene/Toluene	+	90
NIC	Nicotine/Nicotinate	+	N.D.[c]
pJP1	2,4-Dichlorophenoxyacetic acid	+	58
pAC21	p-Chlorobiphenyl	+	65
pAC25	3-Chlorobenzoate	+	N.D.

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[a] *Conjugative plasmids are denoted as +, non-conjugative ones as -.*

[b] *Exact size (daltons) unknown.*

[c] *N.D., not determined.*

SYNTHETIC CHLORINATED HYDROCARBONS: PROBLEMS OF TOXICITY AND PERSISTENCE

The need for continued agricultural productivity and material comforts, as well as increased industrial activities of man, has led to the manufacture of increasing numbers of halogenated hydrocarbons which have been, and are continually being, released into the environment in large quantities. Thus, various chlorinated organic compounds such as 2,4-D (2,4-dichlorophenoxyacetic acid), dalapon (2,2-dichloropropionic acid) and chloramben (3-amino-2,5-dichlorobenzoic acid) have been used as herbicides and pesticides, and have resulted in a phenomenal rise in the agricultural productivity in this country. However, insecticides and pesticides such as DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane], 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) and kepone (Deca-chloro-octahydro-1,3,4-metheno-(2H-cyclobuta-[cd]-pentalene-2-one) have proved to be extremely persistent in the environment and tend to accumulate into animal adipose tissues through food chains. The toxicological problems associated with synthetic chlorinated hydrocarbons such as DDT, 2,4,5-T or kepone are well known and are usually manifested as thinning of eggshells in birds, or damaged liver, testes or nervous systems in animals (2). Similarly, polychlorinated biphenyls (PCBs) are a group of chlorinated hydrocarbons that are widely used in industry as lubricants, heat transfer fluid, and in the protective coatings for wood, metal and concrete. Despite the fact that PCBs have been used for forty years, they were not identified as environmental pollutants until 1966 in Sweden and 1967 in the United States. PCBs tend to accumulate in human tissues through food chains, and have been shown to cause lesions of the gastric mucosa, accumulation of fat in the liver and liver cell necrosis in many animals including man, monkeys and rats (3,4). Because of the widespread occurrence of PCBs in the global ecosystem, their persistence and bioaccumulation in food chains, and toxicity to animals, the U.S. Environmental Protection Agency has issued stringent effluent standards for the point discharge of PCBs by industrial users (5).

The contamination of livestock and the human population in Michigan beginning in July 1973 which resulted in the accidental introduction of polybrominated biphenyls (PBBs, Firemaster FF1) into cattle feed, emphasizes the existent potential for environmental hazard to human health by another group of halogenated hydrocarbons. PBBs have been widely used as fire retardants and the exposure to PBBs may result in abnormalities in reproduction, growth, tolerance of stress, nerve functions, thyroid and kidney functions, etc. (6). Since such compounds are now widespread in the environment, some mechanisms must be found for their removal from contaminated animals, as well as ultimately from the environment.

MICROBIAL BIODEGRADATION OF CHLORINATED HYDROCARBONS

As discussed above, synthetic compounds containing carbon-chlorine bonds are in widespread use and their persistence in the environment has become a matter of considerable concern. There is, however, growing evidence that microorganisms are developing the competence to utilize many chlorinated compounds as a sole source of carbon. Thus herbicides such as 2,4-D or dalapon are known to be biodegradable (7). Enzymes capable of dehalogenating organo-halogen compounds have been purified and studied (8,9).

The presence of part of the metabolic routes in various microorganisms for the degradation of chlorinated hydrocarbon pesticides is of utmost importance in the natural ecosystem and is generally manifested by a process termed cometabolism. Pure cultures can convert insecticides such as DDT to DDD (10) or aldrin to dieldrin (11) but cannot metabolize them all the way to CO_2 and water. Successive enrichment of such pesticides on soils have provided mixed cultures of bacteria which can use aldrin or dieldrin as a sole source of carbon and energy (12). Mixed cultures of bacteria are known to further degrade DDT to simpler metabolites such as DDNS [2,2 bis(p-chlorophenyl)ethane], and pure cultures are known that can degrade partially chlorinated degradative products of DDT such as p,p'-dichlorodiphenyl methane but cannot degrade the parent DDT molecule (13). More recently, single cultures of *P. putida* have also been reported to cometabolize various chlorinated compounds, including DDM (bis-p-chlorophenyl methane), a metabolite generated from DDT (14). Thus cometabolism of DDT among mixed cultures usually leads to its slow degradation in the natural ecosystem. In addition, there is some evidence that microorganisms are evolving new degradative competence for the metabolism of highly chlorinated compounds. The degradation of the herbicide 2-(2,4,5-trichlorophenoxy)propionic acid (Silvex), which is structurally analogous to 2,4,5-T, by a mixed culture of a *Pseudomonas* sp. and an *Achromobacter* sp. has recently been reported (15).

In contrast, highly halogenated compounds such as Kepone or PBBs are not known to be metabolized to any significant extent by the soil microflora. The situation is interesting with respect to PCBs, where significant microbial decomposition can be demonstrated for biphenyl rings containing 1 or 2 chlorine atoms, but not for highly chlorinated isomers. Ahmed and Focht (16) studied the pattern of degradation of several monochloro and dichlorobiphenyl with two species of *Acromobacter* and found that chlorinated benzoic acids formed as a result of oxidative degradation activities on these pCB components. Kaiser and Wong (17) reported the microbial conversion of Aroclor 1242 to reduced non-chlorine containing aliphatic and aromatic degradation products. Using radiolabeled PCBs, Furukawa and Matsumara demonstrated the oxidative metabolism of 2,4,5,2',5'-pentachlorobiphenyl in the presence of biphenyl as a sole source of carbon (18). Sayler, Shon and Colwell (19) have recently reported on the bioaccumulation and degradation of Aroclor 1254 and the uniformly ^{14}C-labeled pure isomer 2,4,5,2',4',5'-hexachlorobiphenyl. The metabolism of 2,4,4'-trichlorobiphenyl by an *Acinetobacter* species has also recently been reported (K. Furukawa, personal communications). These results are encouraging because they indicate that certain microbial strains may have or are evolving efficient metabolic pathways for the degradation of highly chlorinated biphenyls.

SCOPE OF EVOLUTION OF NEW METABOLIC FUNCTIONS IN BACTERIA

It appears that the introduction of large quantities of toxic chlorinated hydrocarbons into the environment has elicited a response from the microbial community in the form of development of new degradative functions against such compounds. This is perhaps similar to the emergence of antibiotic modifying enzymes in various microorganisms, which are believed to have evolved principally as detoxification mechanisms against the widespread use of antibiotics. There is an important difference in the evolution of detoxification mechanisms against antibiotics as against toxic hazardous man-made compounds. Thus, for antibiotic resistance, a bacterium basically needs to evolve a single enzyme that will modify the antibiotic and render it inactive. For detoxification against toxic chlorinated hydrocarbons, the bacterium needs a number of enzymes that will convert the hydrocarbon through a series of intermediate steps to a metabolite which can then enter into the general metabolic pool of the bacterium. It is thus often convenient for microbiota in the natural ecosystem to develop small portions of a given pathway in different microorganisms which must participate as mixed cultures to completely degrade the compound. Because of various constraints on the growth and development of individual cultures in a fixed environment, degradation of highly chlorinated compounds by a mixed culture is usually a slow process. Absence of one or more cultures in the population leads to partial conversion of the parent compound to some intermediates which tend to accumulate. Such intermediates may be acted upon when the appropriate microorganisms become available to the population. Thus cometabolism of the parent toxic compound is a widely-observed phenomenon in microbial degradation of chlorinated hydrocarbons (7,20).

The selective pressure exerted upon the microorganisms to come up with rapid and complete degradation of toxic chlorinated hydrocarbons must have been strong, since pure cultures are also known that can degrade chlorinated hydrocarbons such as 2,4-D or dalapon (7,21). In general, evolution of enzyme systems capable of biodegradation of a man-made compound may occur by two principal modes (22). The enzymes responsible for degradation of naturally-occurring compounds often have broad specificities and may act on synthetic compounds as well as their natural substrates. Alternatively enzyme systems involved in the biodegradation of natural products may act specifically on man-made compounds that are structurally analogous to the natural substrate. The acquisition of simple dehalogenation steps to convert the synthetic compounds into their non-chlorinated derivatives does not appear to be the primary mode of evolution of pathways for the biodegradation of such compounds. Thus a strain of *Alcaligenes paradoxus*, capable of growing with 2,4-D is unable to utilize phenoxyacetic acid (23); similarly *Klebsiella* or *Serratia* species (24), capable of growth slowly with p-chlorobiphenyl are totally incapable of utilizing biphenyl.

The evolution of plasmids encoding partial bioconversion steps without dehalogenation activities appears to be quite common. There are three lines of evidence that suggest that the *Klebsiella pneumoniae* strain AC901 can convert *p*-chlorobiphenyl to p-chlorobenzoic acid, but not beyond this stage. As previously demonstrated (24), growth of *K. pneumoniae* AC901 with p-chlorobiphenyl leads to an accumulation of p-chlorobenzoic acid, which can be detected by gas-chromatography and mass spectrometry. Strain AC901 is also incapable of utilizing *p*-chlorobenzoic acid as a sole source of carbon and energy. Thirdly, there is no liberation of chloride ions during growth of AC901 with p-chlorobiphenyl. All the evidence therefore suggests that the pCB degradative plasmid (pAC21) specifies an incomplete pathway of the bioconversion of *p*-chlorobiphenyl to *p*-chlorobenzoic acid.

Since the 2,4-D degradative plasmid (pJP1) codes for the partial conversion of 2,4-dichlorophenoxyacetic acid to 2,4-dichlorophenol (23), it might be argued that plasmids encoding dechlorination steps have not yet evolved in nature. Reineke and Knackmuss (25) recently presented evidence to suggest that a plasmid may control the complete degradation of chlorobenzoate. We have recently isolated a strain of *P. putida* from sewage that can utilize 3-chlorobenzoic acid (3Cba) as a sole source of carbon with a doubling time of about 170 min. It has been possible to isolate 3Cba-negative segregants that can no longer utilize 3Cba as a sole source of carbon. The results in Table 2 indicate that the 3-chlorobenzoate degradative plasmid (pAC25) is transmissible from the original *P. putida* strain to the 3Cba$^-$ segregants. In addition, pAC25 can be transferred from the original strain to other *P. putida* strains such as PpG1 (AC10) and also to *P. aeruginosa* strain PAO (AC590, Table 2).

To determine whether pAC25 may simply code for a dehalogenase to convert 3-Cba to benzoic acid which can then be utilized by the chromosomally-coded *ortho* pathway, or for a complete 3Cba degradative pathway, we transferred pAC25 to various mutants unable to metabolise benzoate (Ben$^-$) or benzoate and p-hydroxybenzoate (Ben$^-$Pob$^-$) and studied the ability of such 3Cba-positive cells to utilize benozate and p-hydroxybenzoate. As shown in Table 2, transfer of pAC25 to a Ben$^-$Pob$^-$ mutant did not restore the Ben$^+$ or Pob$^+$ phenotype, but the transconjugants were perfectly capable of growing with 3Cba as a sole source of carbon. No growth with benzoate or p-hydroxybenzoate was observed in the presence of inducing quantities of 3Cba. It thus appears that pAC25 specifies a separate but complete chlorobenzoate degradative pathway. It would be interesting to examine the regulation of this pathway and the

Table 2. Transmissibility of pAC25 encoding 3-chlorobenzoate degradation to Pseudomonas *species*

Donor	*Recipient*	*Recipient phenotype*[a]	*Select*	*Frequency*	*Transconjugant phenotype*
AC860 (Ade$^-$ 3Cba$^+$ P. putida*)*	*AC859*	*3Cba$^-$ WT segregant*	*3Cba$^+$*	*2x10^{-6}*	*3Cba$^+$*
	AC1209	*Ben$^-$Pob$^-$*	*3Cba$^+$*	*1x10^{-7}*	*3Cba$^+$Ben$^-$Pob$^-$*
	AC10	*Met$^-$*	*3Cba$^+$*	*1x10^{-8}*	*3Cba$^+$Met$^-$*
	AC590[b]	*Trp$^-$Res$^-$ Sm^r*	*3Cba$^+$*	*1x10^{-7}*	*3Cba$^+$Trp$^-$Res$^-$Sm^r*

[a] *Symbols used: 3Cba, 3-chlorobenzoic acid; Ade, adenine; Ben, benzoate; Pob, p-hydroxybenzoate; Met, methionine; Trp, tryptophan; Res, restriction; Sm^r, streptomycin-resistant.*

[b] *AC590 is a restriction-negative strain of* P. aeruginosa *(PA0403).*

nature of enzymes specified by this plasmid for comparative studies with chromosomally-coded p-hydroxybenzoate or benzoate-degradative enzymes.

CONCLUDING REMARKS

The emergence of plasmids specifying partial or complete dissimilation of such synthetic compounds as 2,4-dichlorophenoxyacetic acid, chlorobenzoic acid or p-chlorobiphenyl demonstrates the versatility of solid microorganisms in coping with the multitude of man-made compounds released into the environment during the last few decades. While the presence of a plasmid encoding complete biodegradation of a chlorinated compound allows the ready removal of that compound from the environment, the presence of a plasmid allowing partial bioconversion does not lead to such removal. Yet, partial bioconversion may be quite advantageous for the microorganisms in a particular ecological niche. For example, the conversion of p-chlorobiphenyl to p-chlorobenzoic acid, coded by the plasmid pAC21, may allow continuous removal of insoluble PCBs from the river bottom sediments because of the water solubility of the product chlorobenzoic acids. The natural occurrence of plasmids such as pAC25 specifying biodegradation of chlorobenzoates suggests that several plasmids are presumably needed for the complete biodegradation of chlorobiphenyls in the environment. The transmissible nature of such plasmids, and a study of their mode of evolution, may in the future facilitate the construction of genetically improved strains that could effectively be used for rapid and complete removal of synthetic pollutants from the environment.

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DISCUSSION

Elwell: Recently PCB was dumped along 15 miles of roadside in North Carolina. It may be interesting to take some soil samples from there.

CHAKRABARTY: You would have to wait 30 years.

Elwell: It will still be there in 30 years.

Behme: Does the PCB-degrading *Klebsiella* belong to any particular species?

CHAKRABARTY: Taxonomically the strain is identical to *Klebsiella pneumoniae.*

PLASMID MEDIATED CARBOHYDRATE CATABOLIC ENZYMES AMONG STRAINS OF *Streptococcus lactis*

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Recent reports by McKay and associates suggest that plasmids may play an important role in the metabolism of the group N streptococci. The ability to ferment lactose (1,5,11,12) and citrate (6), as well as the synthesis of proteinases (5, 11), apparently are plasmid-associated traits in some strains and subspecies of *Streptococcus lactis* and *Streptococcus cremoris*. Because we have been interested in the role of plasmids in the metabolism, ecology and taxonomy of the streptococci in general, we have included one group N species, *S. lactis*, in our studies. Some of our results with this species are presented in the following sections.

PHOSPHOENOLPYRUVATE-DEPENDENT SUGAR PHOSPHOTRANSFERASE ACTIVITIES IN *S. lactis*

The metabolism of many carbohydrates by anaerobic and facultative anaerobic bacteria is initiated by a phosphoenolpyruvate-dependent phosphotransferase system (PTS; 15). The primary function of the PTS appears to be the phosphorylation and concomitant translocation of sugars across bacterial membranes. Four proteins are required to catalyze the overall

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reaction. Two, enzyme I and the phosphocarrier protein (HPr), are general proteins involved in the translocation of all PTS sugars. The remaining two proteins, enzyme II and factor III, are sugar specific.

The group N streptococci transport lactose into the cell as glucosyl-β (1,4)-galactoside-6-phosphate (lactose-P) via a lactose-PTS (13,14). McKay and associates showed that Lac$^-$ isolates of *S. lactis* strains were missing enzyme II and factor III of the lactose-PTS (4,13), and that the loss of ability to metabolize lactose was also accompanied by the loss of a specific plasmid (1,5,11). To determine whether PTS activity could be detected for other sugars metabolized by *S. lactis* we used permeabilized cells (3) and a spectrophotometric assay method described by Kornberg and Reeves (8). We detected PEP-dependent phosphorylation of lactose, galactose, sucrose, fructose, glucose and mannose in strains of *S. lactis*. Preliminary screening experiments indicated that these strains harbored between 6 and 11 plasmids each. Two of these strains, DR1251 (ATCC11454) and DR1252 (#354-07 from L.L. McKay), were chosen to determine whether the metabolism of any of the above PTS sugars was plasmid mediated.

PLASMID-MEDIATED LACTOSE AND SUCROSE METABOLISM IN *S. lactis* STRAIN DR1251

S. lactis strain DR1251 was able to grow on all six of the PTS sugars described above. This strain maintained all of these phenotypic traits when cultured at 30^{o}C, but after growth at 37^{o}C for approximately 12 cell doublings in the absence of lactose, 8% of 144 colonies examined had a Lac$^-$ phenotype. This frequency was increased to 38% following growth in the present of 250 μg/ml of acridine orange, and Suc$^-$ clones were obtained at a frequency of 0.03% (>10,000 colonies screened). The Suc$^-$ isolates were also Lac$^-$, presumably because of the high frequency of loss of the Lac$^+$ phenotype. No isolates unable to grow on galactose, fructose, glucose or mannose were obtained, either at elevated temperatures, or following treatment with plasmid curing agents.

The profiles in the agarose gel of plasmid DNA from Lac$^+$ Suc$^+$ strain DR1251, Lac$^-$ Suc$^+$ strain DR1251/1, and Lac$^-$ Suc$^-$ strains DR1251/26 and DR1251/57 are shown in Figure 1. The loss of lactose metabolic activity was correlated with the loss of a 32 Mdal plasmid. The two Lac$^-$ Suc$^-$ isolates had also lost a 28 Mdal plasmid.

Lactose enters the *S. lactis* cell as lactose-P via a lactose-PTS, and is then hydrolyzed, by P-β-galactosidase (14),

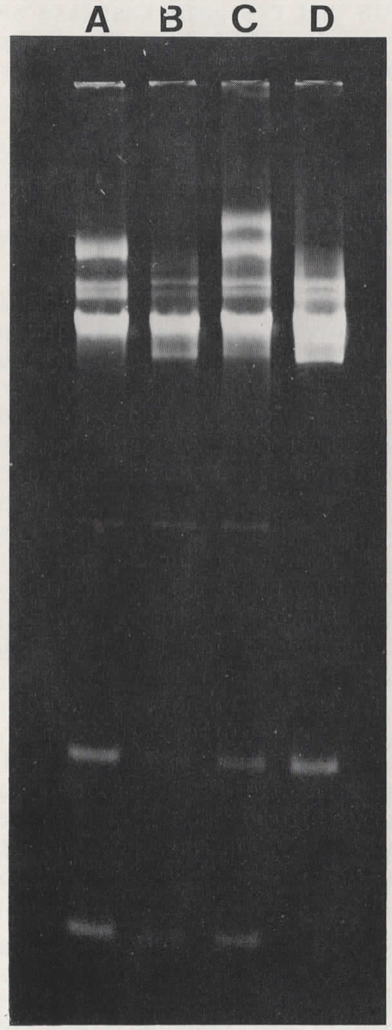

Figure 1. Agarose gel electrophoresis of plasmids from S. lactis *strains DR1251, DR1251/1, DR1251/26 and DR1251/57. Plasmid DNA was extracted from exponential phase cultures by a procedure developed for rapid screening for streptococcal plasmids (LeBlanc and Lee,* J. Bacteriol. 140, *in press). Electrophoresis conditions were as previously described (10). (A) DR1251/1, Lac$^-$ Suc$^+$. (B) DR1251/26, Lac$^-$ Suc$^-$. (C) DR1251, Lac$^+$ Suc$^+$. (D) DR1251/57, Lac$^-$ Suc$^-$.*

to glucose and galactose-6-P, which are further metabolized via the glycolytic and tagatose-6-P pathways, respectively (2, 19). Both lactose-PTS and P-β-galactosidase activities were present in the Lac^{+} strain, DR1251, following growth on either lactose or galactose, but significant activities were not detected in the Lac^{-} strain DR1251/1 (Table 1). Whereas the lactose-PTS was inducible in the parent strain, the P-β-galactosidase appeared to be synthesized constitutively by this strain, as evidenced by the high specific activity observed following growth on D-ribose. Because glucose-PTS activity was present in both strains, the two general PTS proteins, enzyme I and HPr, were still functional in the Lac^{-} isolate.

In a Lac^{-} Suc^{+} strain, DR1251/1, sucrose-PTS activity was induced by growth on sucrose, and in the presence of both sucrose and galactose, but not on either fructose or galactose (Table 2). Sucrose-PTS was not detectable in the Lac^{-} Suc^{-} isolate, strain DR1251/26, under any of the growth conditions tested. Fructose-PTS and the ability to grow on either fructose or galactose were unaffected in the Lac^{-} Suc^{-} strain. The Km value for sucrose in the PTS assay was 6.4×10^{-5} M, suggesting that *S. lactis* strain DR1251 possessed a sucrose-specific PTS system.

INFLUENCE OF THE LACTOSE PLASMID ON THE METABOLISM OF GALACTOSE BY *S. lactis*

McKay and associates (4,13) reported that Lac^{+} and Lac^{-} strains of *S. lactis* grew equally well on glucose and other hexoses, but that Lac^{-} strains always grew poorly on galactose. We also observed that the Lac^{-} strain, DR1251/1, had a longer generation time than the Lac^{+} parent on galactose (Table 3).

Table 1. Enzyme Activities in S. lactis *strain DR1251 and a Lac^{-} Derivative, Strain DR1251/1*

		Sp. Act. in nmoles/min/mg (dry weight of cells)		
Strain	*Growth Substrate*	*Lac-PTS*	*Glu-PTS*	*P-β-galactosidase*
DR1251	10 mM lactose	109	109	594
	20 mM galactose	248	195	360
	20 mM glucose	15	137	21
	25 mM D-Ribose	36	76	549
DR1251/1	20 mM Galactose	0	102	2
	20 mM Glucose	5	57	19
	25 mM D-Ribose	1	55	29

Table 2. Properties of S. lactis *Lac^{-} Suc^{+} Strain DR1251/1, and Lac^{-} Suc^{-} Strain DR1251/26*

			Sp. Act. in nmoles/min/mg (dry weight of cells)	
Strain	*Growth substrate*	*Gener'n time (min)*	*Sucrose-PTS*	*Fructose-PTS*
DR1251/1	10 mM sucrose	40	148	271
	20 mM fructose	45	0	219
	20 mM galactose	82	0	22
	10 mM sucrose + 20 mM galactose	42	121	N.D.[a]
DR1251/26	20 mM fructose	48	0	210
	20 mM galactose	80	0	43
	10 mM sucrose + 20 mM galactose	85	0	N.D.

[a] *N.D. = assay not done*

Bissett and Anderson (2) showed that *S. lactis* possesses the enzymatic potential to metabolize galactose via two pathways. In the first, the tagatose-6-P pathway, galactose enters the cells via a galactose-PTS as galactose-6-P, which is converted to the fructose epimer, tagatose-6-P, and enters the glycolytic pathway at the triose-P level. By the second, or Leloir, pathway a permease mediates transport of the hexose into the cell as free galactose, which is converted to galactose-1-P by galactokinase. Entry into the glycolytic pathway is at the glucose-6-P level. McKay et al. (4,13) suggested that in a Lac^{+} *S. lactis* strain galactose is transported primarily by a PTS, possibly the lactose system, and that metabolism of galactose via the tagatose-6-P pathway may represent the principle pathway for galactose metabolism, with the Leloir pathway operating to a lesser extent. This would also suggest that Lac^{-} strains can use only the Leloir pathway for galactose metabolism. Further studies on strains DR1251 and DR1251/1 provided support for these suggestions (Table 3). Galactose-PTS activity was present in strain DR1251 following growth on either lactose or galactose, but no galactose-PTS activity could be detected in the Lac^{-} strain, DR1251/1, following growth on galactose. Furthermore, when apparent Km values for lactose-PTS and galactose-PTS activities were determined in strain DR1251 these values were approximately 10^{-5} M for lactose as substrate and 10^{-2} M for galactose as substrate, regardless of which of the two carbohydrates was used as the energy source for growth. These results suggested that the sugar-specific components of the lactose-PTS were responsible

Table 3. Galactose Metabolic Enzymes in S. lactis *Strains DR1251 and DR1251/1*

Strain	*Growth substrate*	*Generation time (min)*	*Sp. Act. in nmoles/min/mg (dry weight of cells)*		*Km values*	
			Gal-PTS	*Galactokinase*	*Lac-PTS*	*Gal-PTS*
DR1251	*10 mM lactose*	*42*	*32*	*N.D.*[a]	*4.0x10^{-5}M*	*1.5x10^{-2}M*
	20 mM galactose	*52*	*31*	*825*	*7.0x10^{-5}M*	*2.7x10^{-2}M*
DR1251/1	*20 mM galactose*	*82*	*0*	*3925*	-	-

[a] *N.D. = not done*

for the activities observed on both lactose and galactose in the Lac$^+$ parent strain. In the absence of galactose-PTS activity the Lac$^-$ strain would be expected to metabolize galactose solely via the Leloir pathway. That this was the case was suggested by the 5-fold increase in galactokinase activity in the Lac$^-$ strain following growth on galactose.

PLASMID-ASSOCIATED GLUCOSE AND MANNOSE METABOLISM IN *S. lactis* STRAIN DR1252

Among carboydrate metabolizing facultative bacteria, the ability to ferment glucose is a highly stable property. It was surprising, then, to find that one *S. lactis* strain included in our studies, DR1252, yielded variants exhibiting poor growth on glucose at a high spontaneous frequency (10-40%). When the plasmid profiles of two independently isolated glucose-defective clones, DR1252/703 and DR1252/707, were examined in agarose gels, both were found to be missing a 23 Mdal plasmid species present in the parent strain (Figure 2).

The growth characteristics of the parent strain and a glucose-defective isolate, on other carbohydrates, were compared (Table 4). Whereas the parent strain grew well on glucose, mannose and sucrose, strain DR1252/707 grew much more slowly on both glucose and mannose, and the final growth yield was only half of that observed with the parent strain on these two substrates. The growth rate and final yield on sucrose were the same in both strains.

Table 4. Growth Characteristics of S. lactis *Strains DR1252 and DR1252/707 on Glucose, Mannose and Sucrose*

Strain	*Growth substrate*	*Generation time (min)*	*Final growth yield*[a]
DR1252	*20 mM Glucose*	*45*	*220*
	20 mM Mannose	*50*	*220*
	10 mM Sucrose	*40*	*230*
DR1252/707	*20 mM Glucose*	*120*[b]	*110*
	20 mM Mannose	*120*[b]	*110*
	10 mM Sucrose	*40*	*230*

[a] *Growth in Klett units (#66 filter)*

[b] *Never achieved true exponential phase; calculated as time for doubling of Klett units after growth had begun.*

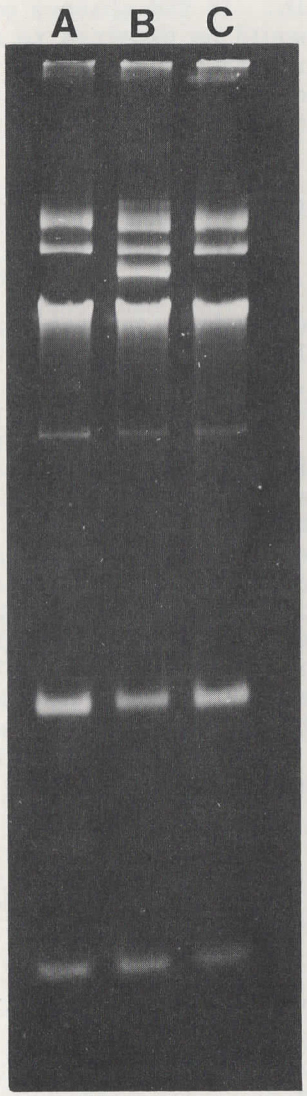

Figure 2. Agarose gel electrophoresis of plasmids from S. lactis *strains DR1252, DR1252/703 and DR1252/707. Plasmid isolation and electrophoresis were as in Figure 1. (A) DR1252/703, Glu^{def}. (B) DR1252, Glu^{+}. (C) DR1252/707, Glu^{def}.*

The glucose-defective and wild-type parent strains have been assayed for PTS activities. The glucose- and mannose-PTS activities in the parent strain were generally 5 to 10-fold lower than the corresponding activities in other strains of *S. lactis*. Results with the glucose-defective isolates of strain DR1252 have been variable. In one experiment the glucose-defective strains had no detectable PTS activity on either glucose or mannose, while in a second experiment the activities were approximately half of those observed in the parent strain on these two substrates. Sucrose PTS activities were similar in the parent strain and in the glucose-defective isolates, and comparable to those observed in other strains of *S. lactis*.

CONCLUSIONS

In addition to the plasmid-associated traits described here, we have found that the loss of a 28 Mdal plasmid by *S. lactis* strain DR1251 resulted in the inability to synthesize the peptide antibiotic, nisin, as well as the inability to metabolize sucrose. In a third strain of *S. lactis*, DR1253, the ability to grow on D-xylose was tentatively correlated with the presence of a 3.6 Mdal plasmid. All of these results suggest that plasmids do, indeed, play a significant role in the metabolism of the group N streptococci.

The interpretations of the results presented in this communication were all based on plasmid curing studies. Obviously, confirmation of the role of plasmids in the metabolic diversity of these streptococci will ultimately depend on genetic transfer studies. We are currently attempting to develop a plasmid transfer system, either conjugation or transformation, for *S. lactis*. A recent report by Kempler and McKay (7) describing conjugational transfer of a lactose plasmid between strains of *S. lactis*, albeit at a very low frequency (10^{-9} per donor cell), is encouraging.

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DISCUSSION

Unidentified: The protoplast system would be worth trying.

LEBLANC: Richard Novick is sending me his system and we're going to try it. If it works in *Staphylococcus*, it probably has a good chance of working with the *Streptococci*.

Vining: I wasn't entirely clear why you got the glucose Pts-cured strain on the sucrose.

LEBLANC: We just grew it on sucrose and checked sucrose Pts to make sure it wasn't a general Pts mutant that we had found.

Vining: How do you account for the ones that have lost the glucose Pts plasmid?

LEBLANC: How did they do it? I haven't the vaguest idea. I'm amazed that it goes so fast. If we leave the wild type culture off glucose for about six transfers, we have trouble getting the wild-type up again. It loses that plasmid at a very rapid rate.

R-PLASMIDS AND NOSOCOMIAL INFECTION[1]

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INTRODUCTION

A nosocomial infection is defined as one which occurs during hospitalization but which was not present or incubating upon admission of the patient to the hospital (4). Infections which occur after a patient has been discharged may also be considered nosocomial if the infecting pathogen is judged to have been acquired during hospitalization. A variety of studies in individual hospitals have contributed to our understanding of these infections but an overview of this problem is best supplied by the surveillance system set up by the Center for Disease Control, the National Nosocomial Infections Study (NNIS), which monitors trends in nosocomial infection rates, pathogens and antimicrobial susceptibility patterns. In this system, 80-100 hospitals of various sizes and locations use standard definitions for infection and provide this information to the CDC for tabulation and analysis.

Hospitals participating in NNIS surveillance during 1975-1976 reported a nosocomial infection rate of 356.6 per 10,000

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patients discharged (7). Although there is some fluctuation in rate from month to month, this overall rate has remained about the same over the past five years. There are significant variations in the reported nosocomial infections rates, however, depending upon the type of hospital and particular specialty services within a given hospital. For example, the nosocomial infection rate for community hospitals (acute care, non-governmental or university hospitals) is about 2.5% while municipal/county hospitals have a rate of about 5.8%. Similarly, the infection rate ranges from 1% on pediatric services to 3.7% and 5.0% on the medical service and surgical service, respectively.

Urinary tract infections are the most common type of nosocomial infection accounting for 40% of the total observed. Surgical wound infections account for 24% of all nosocomial infections, while lower respiratory tract infections comprise about 16%.

Bacteremias which account for about 5% of all nosocomial infections are the most serious since they are associated with the highest fatality rates (see Table 1). Bacteremias reported to NNIS are classified as primary or secondary. Primary bacteremia, defined as bacterial infection of the blood with no other site in that patient infected with the same pathogen before the onset of infection, was reported at a rate of about 17 per 10,000 hospital discharges. Secondary bacteremia, which is an infection of the blood bacteriologically, temporally and clinically related to an infection at another site on the patient occurred at a rate of 15.1. These rates varied by category of hospital, ranging for primary bacteremia from 6.3/10,000 in community hospitals to 28.7/10,000 in municipal hospital. Similarly the rates for secondary bacteremia ranged from 6.3 in 10,000 in community hospitals to 36.9/10,000 in municipal hospitals. It is important to note that secondary bacteremia occurred in about 1 in 30 individuals who developed a nosocomial infection in NNIS hospitals.

Table 1. Nosocomial Infections Caused by Gram Negative Rods (GNR)

Site of infection	*% of all nosocomial infections*	*% caused by GNR*	*% with bacteremia (secondary)*	*Case fatality ratio*
Urinary tract	40	84	1	.01
Surgical wound	24	68	0.6	.04
Respiratory tract	16	74	1.7	.20
Bacteremia (primary)	5	78	-	.34
Other	15	67	*variable*	*variable*

Over 90% of the reported nosocomial infections were examined bacteriologically. Table 2 shows that relatively few groups of microorganisms accounted for about 78% of all isolates. Interestingly as shown in Table 2, the most common organisms causing nosocomial infection are the same as those which are favorite subjects of study by microbial geneticists and molecular biologists. Yet, there has been little research devoted to the biology of the strains isolated from nosocomial infection and to whether they differ from similar strains encountered in the community environment.

ANTIBIOTIC RESISTANCE AND NOSOCOMIAL INFECTION

Nosocomial infection is not new. It has been part of medicine since the earliest times. Yet, there has been a striking change in the relative frequency of occurrence of some of the common pathogenic bacteria as causative agents of serious nosocomial disease. This changing ecology of serious bacterial infections has been particularly well documented by Maxwell Finland and his colleagues at Boston City Hospital (3). One such study concentrated upon bacteremic infections during 12 selected years between 1935 and 1972 (6), and as shown in Table 3, documented the emergence and increasing occurrence of cases caused by Gram negative organisms. Moreover, bacteremias caused by Klebsiella-Enterobacter and Pseudomonas were much more frequent among hospital acquired bacteremias than among those that were acquired in the community.

There are several reasons why Gram negative organisms have emerged as the predominant offending pathogen in hospital infection. Certainly, an important factor is that the patients

Table 2. Organisms Causing Nosocomial Infections[a]

Gram negative		*Gram positive*	
E. coli	*20.9%*	Staphylococcus aureus	*9.6%*
Proteus sp.	*6.9%*	Enterococcus	*7.7%*
Pseudomonas aeruginosa	*8.9%*	Staphylococcus epidermidis	*3.6%*
Klebsiella pneumoniae	*8.5%*	Streptococcus pyogenes	*2.2%*
Enterobacter sp.	*4.7%*	Streptococcus pneumoniae	*1.1%*
Serratia marcescens	*1.1%*		
	53.7%		*26.2%*

Remaining 22.1% due to non-bacterial pathogens (fungi, viruses, protozooans) or to bacterial pathogens not listed.

[a] *As modified from National Nosocomial Infections Study (Ref. 2)*

Table 3. Changes in the Etiology of Bacteremia[a]

Organism	*% of all bacteremia infections* 1935	1972
Streptococcus pneumoniae	32.5	14
Streptococcus pyogenes *(Group A)*	17.2	5
Staphylococcus aureus	21	12
Enterobacteriaceae (E. coli, Proteus, Klebsiella, Enterobacter *and* Pseudomonas)	12	40

[a] *Modified from data presented by Finland (3) and McGowan* et al. *(4)*

at risk are often at the extremes of age and have been subjected to "heroic" surgical and medical measures. A variety of measures necessary for care of their underlying disease such as urinary catheterization, surgical incision, or tracheal intubation will bypass the normal host defense mechanisms such as intact skin, mucous membrane, cough reflex and so on. These patients are often very susceptible to microorganisms that would otherwise be considered of low virulence. Often the offending nosocomial pathogen comes from the patient's own flora and emerges under the selective pressure of antibiotic therapy. The usual scenario is that during antibiotic therapy (whether in or out of hospital) a largely resistant fecal flora emerges. If a patient has a lowered resistance to infection because of instrumentation, immunosuppression or other factors, an infection may develop. It is important to note that in most cases, while the organism is resistant and often carries an R plasmid conferring resistance to the antibiotic used for initial therapy, these infections are generally well contained and readily respond to alternative therapy. In a small proportion of instances, however, (and these are the most serious instances) a multiply-resistant Gram negative species becomes resident within the hospital environment. Under these circumstances, patients receiving antibiotics almost exclusively become colonized on their skin, posterior pharynx and develop intestinal carriage of some species. These patients seem to be most likely to develop a nosocomial infection.

The study of resistant nosocomial organisms has been clouded by several factors. (i) At the clinical level, resistance to tetracycline, sulfonamide, chloramphenicol, in fact many of the 'standard' markers of the "plasmidologist" are often considered less relevant by the clinical laboratory than they would be for community-acquired pathogens. In treatment of serious infections in hospitalized patients the penicillins particularly carbenicillin; the cephalosporins; and the aminoglycosides, gentamicin, tobramycin and amikacin; are most used and thus many attending physicians would not consider that there was a significant "resistance problem" unless one or more of these therapeutic cornerstones was compromised. It has been difficult, therefore, to obtain good genetic and molecular data concerning resistance mediated by R plasmids in nosocomial pathogens until serious outbreaks had already occurred. (ii) There has been a temporal variation in both the resistance pattern of nosocomial pathogens and in the predominant nosocomial pathogen isolated in a particular hospital setting. Thus, it is not uncommon for a particular service within a hospital to first experience a significant number of clustered infections due to one nosocomial pathogen, say *Serratia marcescens*, which gives way to a second cluster of infections due to *Klebsiella pneumoniae; Pseudomonas aeruginosa; Proteus rettgeri;* or *Citrobacter freundii*. All of these organisms may possess significant resistance to carbenicillin or the gentamicin-tobramycin group, or both. The question has been: are these the dissemination of a single plasmid type within the hospital setting, or are these independent episodes? Are the relevant resistance determinants carried on plasmids or in preexisting chromosomal genes? (iii) Multiply resistant nosocomial pathogens differ significantly in the type and severity of infection that they cause. For example, *Klebsiella pneumoniae* in some instances may cause exclusively urinary tract infection with only a low proportion of the patients becoming bacteremic. In other instances, (even in the same clinical setting), *Klebsiella pneumoniae* causes significant respiratory infection and wound infection in addition to urinary tract infection with these infections often going on to bacteremia with significant mortality (9). In a similar vein *Serratia marcescens* strains often cause only asymptomatic urinary tract infection, although in some outbreaks one sees a high incidence of bacteremia and significant mortality. Very little of the pathogenesis of these infections is known. Certainly, the vast majority of nosocomial *Klebsiella pneumoniae* isolates are encapsulated so that the possession of a capsule *per se* is not the only answer. Do strains have significantly different colonization factors such as pili? Could these and other factors such as serious resistance be mediated by plasmids? These are questions that are not yet answered nor even, as far as we know, a current subject of serious study. (iv) Most hospitals have appropriate epidemiological surveillance designed to detect and control nosocomial infection. In addition, the clinical laboratory in all but the smallest hospitals can supply rapid and accurate antimicrobial susceptibility data. Often the problem for the clinician and the epidemiologist is how to interpret the laboratory findings. There has been a natural tendency to group all multi-resistant strains, or at least strains resistant to the gentamicin-tobramycin-amikacin complex, into the same broad category. Of course workers in the R plasmid field now appreciate that the similarity in antibiogram, even in the same species or serogroup of organisms, does not necessarily mean that a common plasmid or even mechanism of resistance is involved.

MOLECULAR EPIDEMIOLOGY OF NOSOCOMIAL OUTBREAKS

Over the past few years we have used some of the more direct means of genetic and molecular plasmid analysis to examine multi-resistant nosocomial isolates and have examined over a dozen distinct nosocomial outbreaks throughout the United States. The full details of these studies will be published elsewhere but we will summarize the salient points for present consideration.

One of the most striking features of our investigations was that agarose gel electrophoresis (AGE) of crude cell lysates together with straightforward genetic crosses often gave us invaluable data on the epidemiological situation within a given clinical setting. The AGE pattern of plasmid DNA is often an excellent epidemiological marker. That is, strains of a nosocomial pathogen show identical AGE patterns that can be distinguished from AGE patterns from strains of the same species, even with the same antibiogram, that are not linked epidemiologically to an outbreak under study. Figure 1 illustrates the point. In this instance, a strain of *Providencia rettgeri* (formally called *Proteus rettgeri*) resistant to gentamicin, kanamycin, sulfonamide, chloramphenicol, and carbenicillin was implicated in a nosocomial outbreak. The issue was somewhat clouded, however, since strains of this species with a similar antibiogram were encountered occasionally from different wards throughout the hospital. The question was whether indeed there was a defined outbreak limited to the intensive care unit, or whether this hospital was on the verge of a general outbreak of nosocomial disease.

The strains of *P. rettgeri* were lysed and anlyzed by AGE. As shown in Figure 1 (A,C,D) epidemic strains which were isolated from the intensive care service possessed but a single high molecular weight plasmid while two *P. rettgeri* strains from the same hospital, but unlinked epidemiologically, possessed readily distinguishable AGE patterns. Subsequent studies showed that the plasmid present in the epidemic strain

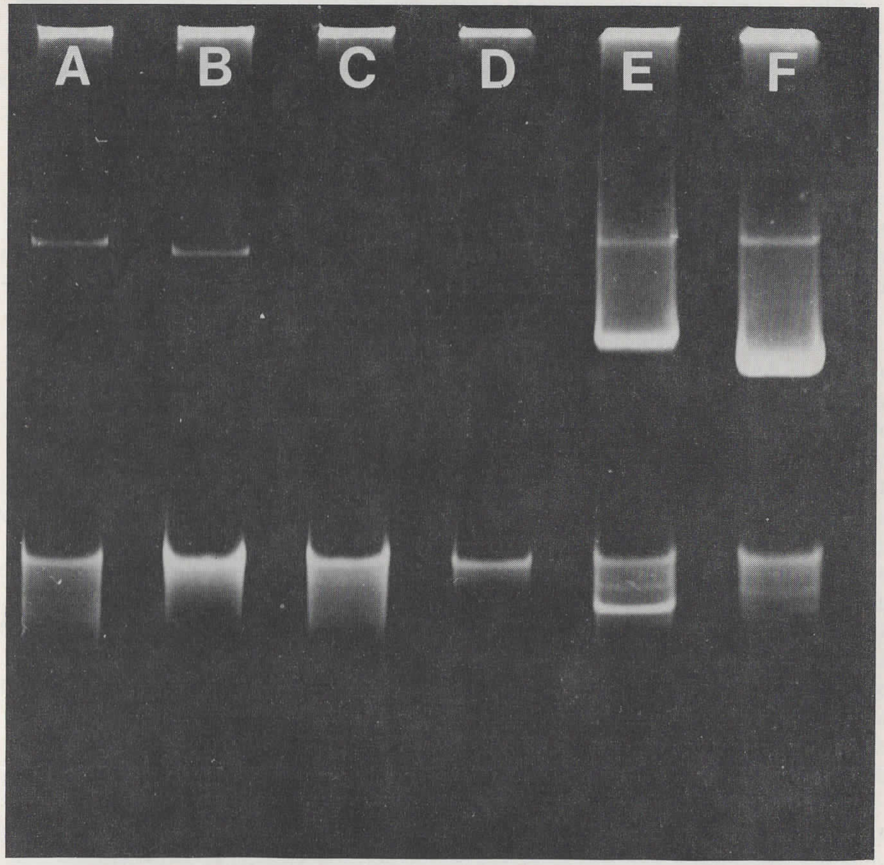

Figure 1. Agarose gel electrophoresis pattern of DNA isolated from resistant Providencia rettgeri *strains. A. Crude DNA lysate of multiply-resistant epidemic isolate of* P. rettgeri, *R-62. B. Crude DNA lysate of* E. coli *K-12 C600 transconjugant from a cross between the epidemic strain and* E. coli *K-12 C600. This plasmid pUW62 mediates resistance to carbenicillin, ampicillin, kanamycin, sulfonamide and chloramphenicol. C and D. Crude lysates of* P. rettgeri *strains R-44 and R-39 epidemiologically and serologically linked to R62. E and F. Crude lysates of* P. rettgeri *strains R-71 and R-25 isolated from the same hospital but from a different epidemiologic setting. The antibiograms of all the strains were similar.*

was transferable to *E. coli* K-12 (Figure 1B) and conferred resistance to all antibiotic markers except gentamicin. Hence, in this case gentamicin resistance was probably mediated by a chromosomal mutation. Using more "standard" epidemiological tools such as serotyping and biotyping we later confirmed the implications of the AGE patterns; that is, the epidemic strains from the intensive care unit were identical, whereas the other *P. rettgeri* strains possessed different markers.

In this instance, as in most of the nosocomial outbreaks we studied, the epidemic had been 'cloned' in origin. There was usually a recognizable strain carrying a distinctive R plasmid or chromosomal markers, or both. As Mark Richmond has appropriately pointed out, plasmids cause epidemics in bacteria much the same as bacteria cause epidemics in people.

We can document that within a given hospital setting an R plasmid may eventually become established and sometimes in a wide variety of bacterial species. Indeed, over a two year period we have followed one R plasmid in seven distinct enteric genera. Figure 2 illustrates the point. In this study strains of both *Klebsiella pneumoniae* and *Enterobacter cloacae* with the same antibiogram were isolated from the same pediatric intensive care unit. Crude lysates of these strains analyzed by AGE established that both shared large plasmids of the same molecular mass (Figure 2C,D). Both could transfer to *E. coli* K-12 a plasmid conferring resistance to gentamicin, tobramycin, kanamycin, carbenicillin, and chloramphenicol. Under the appropriate conditions one can also simply subject purified plasmid DNA to restrictive endonuclease digestion and determine whether they exhibit a similar banding pattern (Figure 2A,B, E and F).

The usefulness of the AGE approach is particularly apparent when one examines species such as *Citrobacter freundii* for which no readily available typing scheme exists. Even well established typing schemes, for example capsular typing of *K. pneumoniae*, may not provide such readily available information as does the AGE method. That is, serotyping will show when two organisms are potentially related but the AGE method, particularly when coupled with restriction endonuclease analysis can provide preliminary evidence for the similarity of strains on the basis of plasmid identity. The use of genetic crosses to determine whether resistance is plasmid-mediated and whether there is more than one plasmid involved also provides important information. Indeed, the genetic and molecular approach to the study of organisms causing nosocomial infection can provide the most rapid and informative data to attack this type of epidemic problem. At the practical level it is of particular importance to determine whether one is dealing with sporadic instances of multi-resistance or whether there is a *bona fide* nosocomial

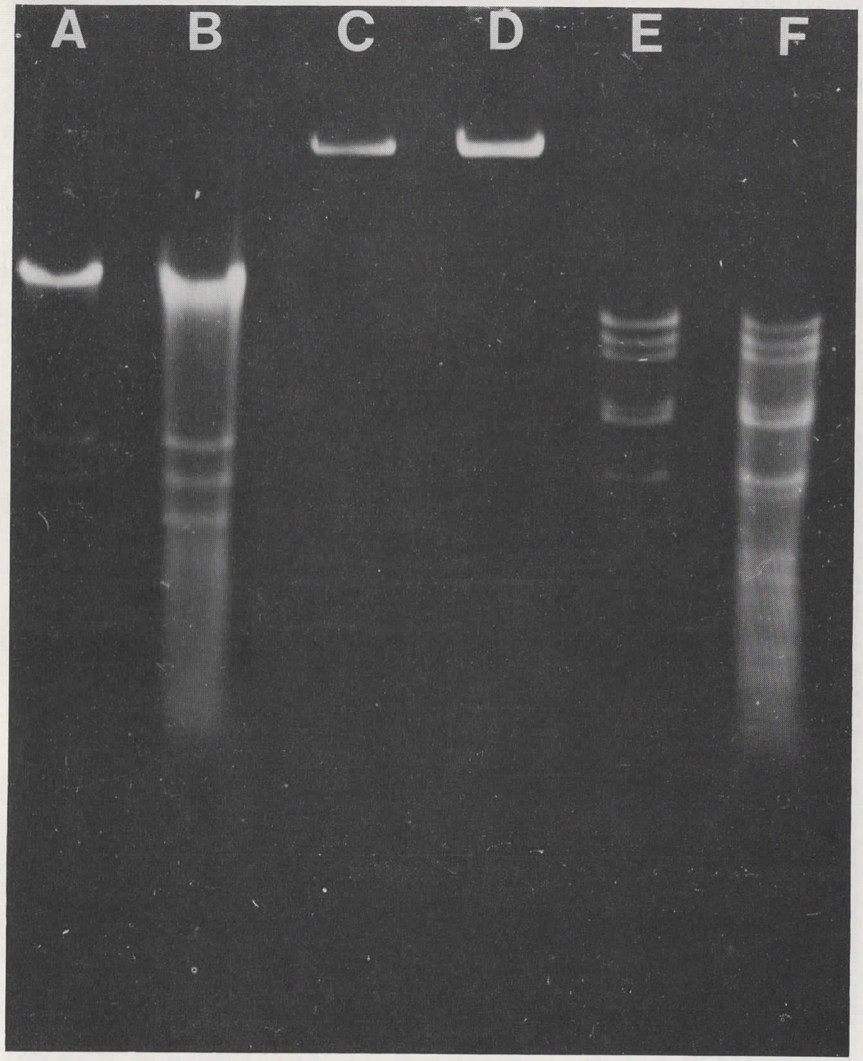

Figure 2. The dissemination of a single R plasmid among different enteric species. C and D. DNA from plasmids isolated from Klebsiella pneumoniae *and* Enterobacter cloacae *with the same antibiogram isolated from the same pediatric intensive care unit. Purified plasmid DNA from the* K. pneumoniae *strain, pUW1 and* E. cloacae, *pUW21 were cleaved with the restriction endonuclease EcoRI (A and B) as well as* Bam*HI (E,F).*

outbreak since the control measures one applies will vary accordingly.

THE BIOLOGY OF PLASMIDS ASSOCIATED WITH NOSOCOMIAL OUTBREAKS

Very little is known concerning the biology of the plasmids associated with nosocomial infection. Incompatibility testing has generally been applied to only single outbreaks (1). Thus, it is not clear, for example, whether one is dealing with plasmids which are confined within specific local situations or if indeed there is a world-wide dissemination of but a few plasmid types within the hospital setting. These questions are readily amenable to study, however, so that we may expect to find the answers in the not too distant future. Certainly, our data showing the rapid dissemination of a single plasmid in many genera, suggests that plasmids isolated from nosocomial pathogens are often of broad host range.

Despite the very fundamental information that is lacking one can show that the molecular evolution of plasmids is dynamic in the hospital setting under the extraordinary antibiotic pressure that is applied. The study of these dynamic changes becomes attainable once the plasmid 'baseline' has been determined within a given clinical setting. Accordingly, one of the more interesting questions is whether transposons determining resistance to antibiotics are as actively at play in nature as our *in vitro* studies lead us to believe. If one examines plasmids isolated from nosocomial outbreaks from several geographical locations it can be shown that those mediating carbenicillin resistance, with few exceptions, possess a Tn*1* or Tn*3* transposon element (5). Yet, one should like to observe the actual accretion of one or more transposons under selective pressure.

Recent studies by Craig Rubens and his associates at the Medical University of South Carolina and Vanderbilt University have documented molecular reassortment of plasmids during an ongoing nosocomial outbreak that involved four different hospitals (8), inter-hospital spread apparently taking place via the hands of the medical staff (9). In strains of *S. marcescens* and *P. aeruginosa* isolated early in the outbreak, resistance to gentamicin and tobramycin was mediated by a common 9.8×10^6 dalton non-conjugative plasmid. In the *S. marcescens* strains there was an additional 80×10^6 dalton or 100×10^6 dalton conjugative plasmid which conferred resistance to several other antimicrobial agents. In gentamicin (tobramycin)-resistant strains of *S. marcescens* and *K. pneumoniae* isolated later in the epidemic, a single 106×10^6 dalton plasmid was found. This latter plasmid was shown to be a composite of the coexisting *S. marcescens* plasmids in which a 6.2×10^6 dalton DNA segment conferring resistance to gentamicin (tobramycin), kanamycin, ampicillin, and carbenicillin had transposed from the 9.8×10^6 dalton plasmid to the 100×10^6 dalton conjugative plasmid, resulting in the formation of a conjugative plasmid which possessed a full complement of resistance markers. The evolution of this conjugative plasmid surely was responsible in part for the broad dissemination of multiple antibiotic resistance in the *S. marcescens*, *K. pneumoniae*, and *E. cloacae* epidemic. Moreover, it is clear that this single transposon possesses sufficient genetic information to render useless the major therapeutic resources (with the exception of amikacin) employed for the treatment of serious nosocomial infections caused by Gram negative organisms.

Unquestionably other instances of transposition *in vivo* occur and can be documented (2). The study of R plasmids which inhabit nosocomial pathogens as well as an appreciation for the epidemiological factors contributing to plasmid-spread offer an extraordinary opportunity for the microbial geneticist and molecular biologist to examine plasmid biology, as it were, in its natural habitat.

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DISCUSSION

Bryan: Most of your nosocomial strains appear to have urinary tract origins, is that correct?

FALKOW: Yes.

Bryan: In studies that have been made, particularly relating to *Pseudomonas,* it is of interest that reservoirs are almost always topical or urinary infections. There are rarely systemic or pulmonary infections, is that correct?

FALKOW: It's certainly true in the case of *Serratia marcescens* as well. I think *Serratia* and *Pseudomonas* behave similarly but in the case of *Klebsiella* it appears that some have specifice adherence factors that permit them to colonize quite well, and their primary reservoir is the bowel. A group in Denver as well as ourselves and others have observed that the longer a patient stays in the hospital, the higher the risk of *Klebsiella* infection. If you follow the process over a period of time, supposing that there is a hospital strain of *Klebsiella* circulating about, then patients often become gut carriers of this *Klebsiella* and many of them have it as long as they stay in the hospital. It usually disappears after they go home, but a definite percentage of those patients will develop either pneumonia or urinary tract infections from their intestinal colonisation. So I think *Klebsiella* is different. As for *Citrobacter,* I really don't know where the reservoir is and in our hands that's becoming more and more important and there I believe it's going to be intestinal colonisation. I think the reservoir will vary depending on the colonising ability of these organisms. This has not been studied in detail, although we've done a study and clearly we can show, at least for *Klebsiella,* that some strains show very good colonisation.

Kopecko: Since nalidixic acid and nitrofurantoins are being used heavily for urinary tract infections, have you come across plasmid mediated resistance to these?

FALKOW: No.

Kopecko: Do you have any thoughts on that?

FALKOW: I have lots of thoughts on that but no data. For many of the "products of the chemists bench" you often don't see plasmid mediated resistance. Those are the two primary examples in clinical medicine of fully synthetic compounds that are used successfully in which one sees no plasmid mediated resistance. In a sense that could be looked at as being support for Julian Davies' idea of where the genes for these things come from. I should also say that we see a steady creeping upwards of resistance in strains coming from the community at large but nothing too remarkable, except in sulfonamide resistance and of course that's used widely for out-patients who suffer urinary tract infections. Where one only used to see at one point 10-15% resistance in the community, that's now up close to 25-30% resistance and I attribute largely to this the fact that ampicillin is beginning to replace sulfur in the community in the treatment of primary urinary tract infections.

MECHANISMS OF PLASMID MEDIATED DRUG RESISTANCE

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Plasmid specified resistance to antimicrobial agents has become the most widespread mechanism of bacterial resistance to antibiotics. However, with the development of an understanding of insertion elements and transposable DNA, the distinction between plasmid mediated and chromosomally mediated resistance has become less clear. It seems reasonable to assume that most, if not all resistance genes can appear as components of plasmids by way of transposable DNA. However, it is clear that, for the most part, there is a distinction between plasmid mediated and chromosomally mediated resistance mechanisms. This distinction is probably due to the fact that plasmid mediated resistance must have a dominant effect in order to be expressed. Plasmid specified resistance is mainly associated with the acquisition of an additional property usually in the form of an enzyme or enzymes. In general terms these enzymes modify antibiotics, circumvent the target, or result in reduced accumulation of the antibiotic. Chromosomal resistance generally results only in alteration of target sites. Macrolide-lincosamide resistance is an exception; it is caused by plasmid specified alteration of the target.

Tables 1 and 2 are lists of antibiotic resistances which have been generally associated with plasmids or with chromosomal genes respectively.

A. RESISTANCE TO β-LACTAM ANTIBIOTICS

Resistance results from enzymic hydrolysis of the β-lactam ring of penicillins and cephalosporins. Plasmid mediated

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Table 1. Antimicrobial Agents for Which Plasmid Mediated Resistance Has Been Reported

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Penicillins
Cephalosporins

Aminoglycosides
Spectinomycin

Tetracyclines

Chloramphenicol
Fusidic acid
Novobiocin

Trimethoprim

Sulfonamides

Macrolides
Lincomycins

Heavy metals - mercury and organomercurials
  - cadmium
  - silver
  - arsenate, arsenite
  - bismuth
  cobalt
  others

Table 2. Antibiotics for Which Plasmid-Mediated Resistance Has Not Been Reported[a]

Vancomycin
Cycloserine
Phosphonomycin
Bacitracin
Gramacidin
Polymyxins
Nalidixic acid
Nitrofurans
Isoniazid

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[a] *Although plasmid-mediated β-lactamases hydrolyzing β-lactamase resistant penicillins like methicillin have been reported, they have not been shown to mediate clinically important methicillin resistance.*

resistance to the latter is very much less common than the former.

Various classifications of β-lactamases have been prepared. Most classification schemes involve enzymes from Gram-negative bacteria. Factors used to identify enzymes include the profile of substrates hydrolyzed, presence or absence of inhibition by cloxacillin or p-chloro-mercuribenzoate, molecular weight, isoelectric point and inducible or constitutive genetic control. The commonest β-lactamases specified by plasmids are the TEM-1 and TEM-2 enzymes. The wide distribution of such genes is accounted for by their presence on Transposon A (TnA=Tn1, Tn2 or Tn3) and presence of TnA on plasmids with wide host ranges.

The number of different plasmid specified β-lactamases is probably very large. In a recent review Sykes and Matthew (20) described five types: TEM-1, TEM-2, OXA-1, OXA-2 and OXA-3. However, it is likely that many additional enzymes exist. For example, Jacoby and Matthew (10) showed that four enzymes not belonging to the above groups are present on *Pseudomonas* plasmids (PSE-1, PSE-2, PSE-3, PSE-4). In addition, in reviewing *Pseudomonas aeruginosa* enzymes (4), I found two further enzymes that were apparently different from all of the above (PCase II, PCase I). This still leaves enzymes from other bacteria particularly those from staphylococci which are not included in the above list.[1]

Resistance to β-lactams is by detoxification. However, this is achieved in different ways by different bacteria (20, 18). Gram-positive bacteria like *Staphylococcus aureus* liberate the β-lactamase into the growth medium and steadily destroy susceptible β-lactam molecules present (Figure 1). The level of resistance will depend on the amount of β-lactamase activity available which in turn depends on the number of bacteria (hence is associated with a pronounced inoculum effect) and the amount of enzyme per cell. β-lactamases from Gram-positive bacteria have high affinities for β-lactam antibiotics (low Km's).

In Gram-negative bacteria like *Escherichia coli*, most enzymic destruction of β-lactams occurs in the periplasmic space (Figure 2). Under these circumstances, the level of resistance depends on the rate at which β-lactam molecules penetrate the outer membrane, and the rate of hydrolysis. The latter is a function of the Km, Vmax, and to some extent the total amount of enzyme per cell. The rates of outer membrane penetration differ depending on the type of β-lactam (e.g., cephaloridine penetrates the *E. coli* membrane more readily than

[1] *At least 11 types of β-lactamase exist among Gram-negative bacteria including types HMS-1 and SHV-1, all of which can be differentiated by isoelectric focussing.*

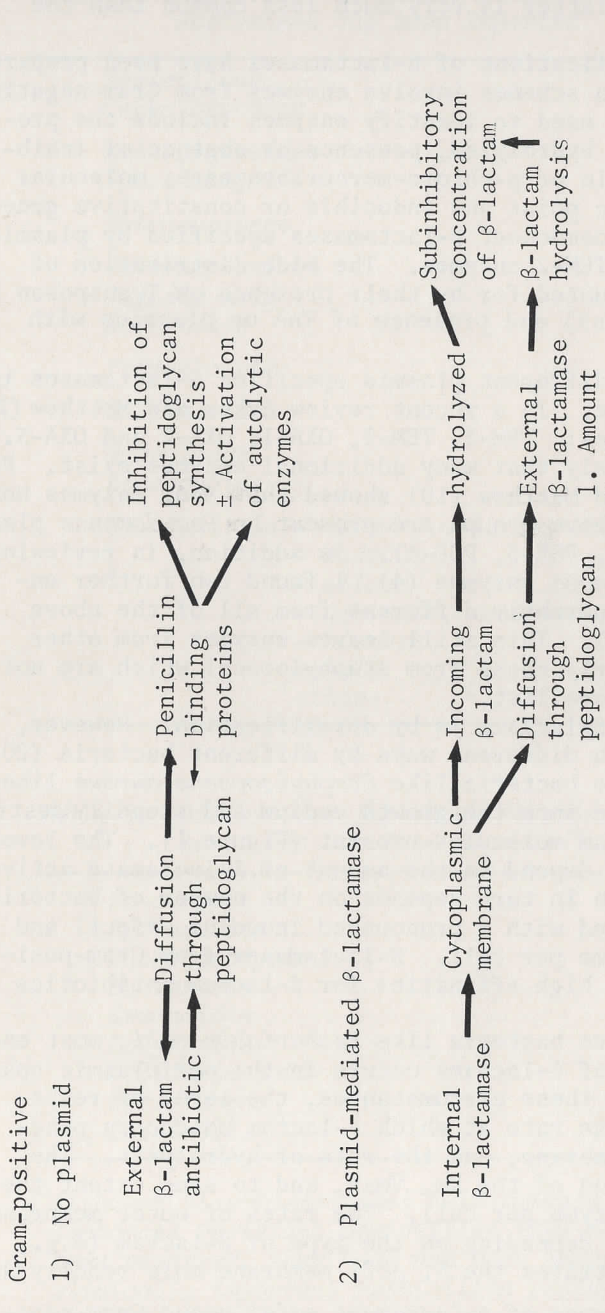

Gram-positive
1) No plasmid
External β-lactam antibiotic
Diffusion through peptidoglycan
Penicillin binding proteins
Inhibition of peptidoglycan synthesis ± Activation of autolytic enzymes
2) Plasmid-mediated β-lactamase
Internal β-lactamase
Cytoplasmic membrane
Incoming β-lactam
hydrolyzed
Subinhibitory concentration of β-lactam
Diffusion through peptidoglycan
External β-lactamase
1. Amount
2. Km
3. Vmax
β-lactam hydrolysis

Figure 1. Plasmid-specified resistance to β-lactam antibiotics in bacteria. 1) No plasmid. The route of entry of a β-lactam to target sites (penicillin-binding proteins) is shown. 2) Plasmid. The release of cellular β-lactamase molecules to hydrolyze incoming β-lactam molecules within the cell wall and in the medium.

penicillin G), the concentration in the medium, and the type of bacterium. Apparently the outer membrane of some Gram-negative bacteria, like *Hemophilus influenzae* poses a much lower penetration barrier to many β-lactams than does that of *E. coli*.

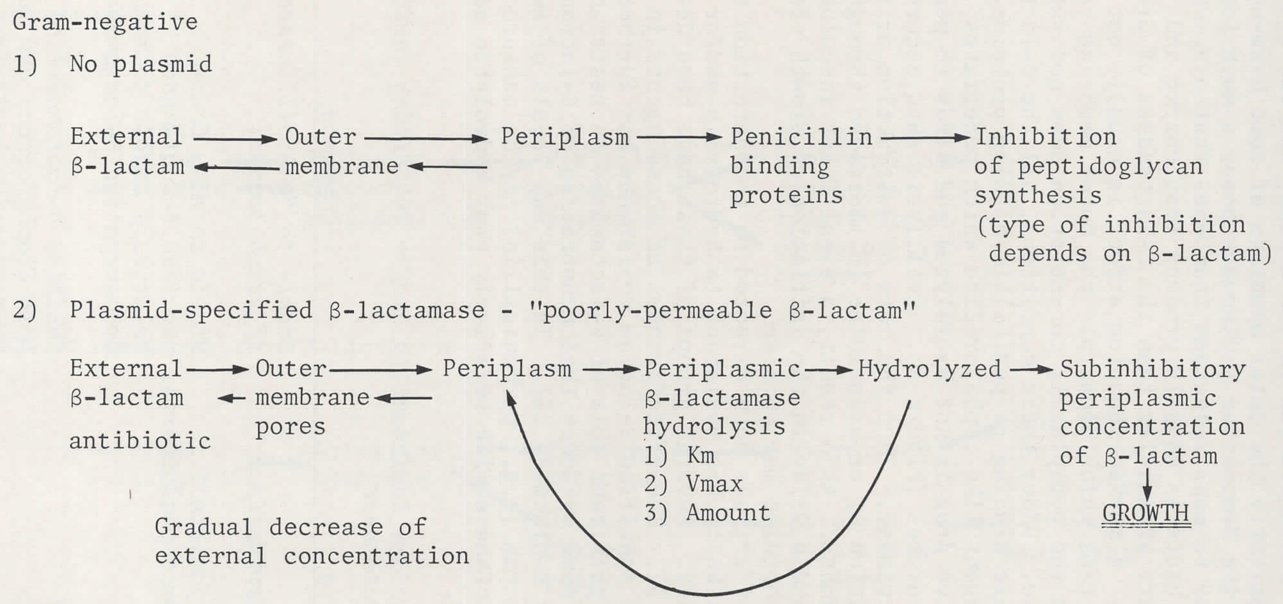
Gram-negative bacteria usually produce less enzyme and with lower substrate affinities than the β-lactamases of Gram-positive bacteria. A marked inoculum effect is usually not detected for β-lactams which penetrate the outer membrane relatively poorly as the periplasmic conentration does not saturate the β-lactamase. Under these conditions, all the β-lactam is hydrolyzed before binding to penicillin binding proteins. However, with an agent like cephaloridine which penetrates readily, some active drug is not hydrolyzed and binds to penicillin-sensitive enzymes (Figure 2). Cell lysis then occurs and releases β-lactamase. Thus the rate of destruction of the external concentration of cephaloridine is increased through release of β-lactamase. As a result, a significant inoculum effect is likely and a delay in the initiation of growth after addition of cephaloridine may be seen.

Considerable effort has been directed to the detection of β-lactam molecules which are resistant to hydrolysis and/or inhibit β-lactamases. Several groups of these have been detected (see Table 3). The effectiveness of these agents in overcoming plasmid specified β-lactam resistance has further illustrated the significant role of β-lactamases in resistance.

There is also some evidence that penetration of β-lactams can be impaired by R-plasmids (6). In this way levels of resistance to a β-lactam (e.g., carbenicillin) may be enhanced in spite of a β-lactamase with relatively poor hydrolytic activity on the drug.

Table 3. β-Lactams Resistant to and/or Inhibiting Various β-Lactamases

Agents	*Comments*
1) Methicillin Oxicillin and derivatives Nafcillin	*Stable to "penicillinases" of staphylococci*
2) Cefoxitin (a Cefamycin) LY127935 (a 1-Oxacephalosporis)	*Stable to many Gram-negative β-lactamases*
3) Cefamandole Cefuroxime Cefotaxime	*Relatively stable to many Gram-negative β-lactamases*
4) Clavulinic acid	*Inhibits β-lactamases of both Staphylococci and many Gram-negative bacteria*
5) Thienamycin	*Inhibits β-lactamases and has broad spectrum antimicrobial activity*


Gram-negative
1) No plasmid
External β-lactam
Outer membrane
Periplasm
Penicillin binding proteins
Inhibition of peptidoglycan synthesis (type of inhibition depends on β-lactam)
2) Plasmid-specified β-lactamase - "poorly-permeable β-lactam"
External β-lactam antibiotic
Outer membrane pores
Periplasm
Periplasmic β-lactamase hydrolysis
1) Km
2) Vmax
3) Amount
Hydrolyzed
Subinhibitory periplasmic concentration of β-lactam
GROWTH
Gradual decrease of external concentration

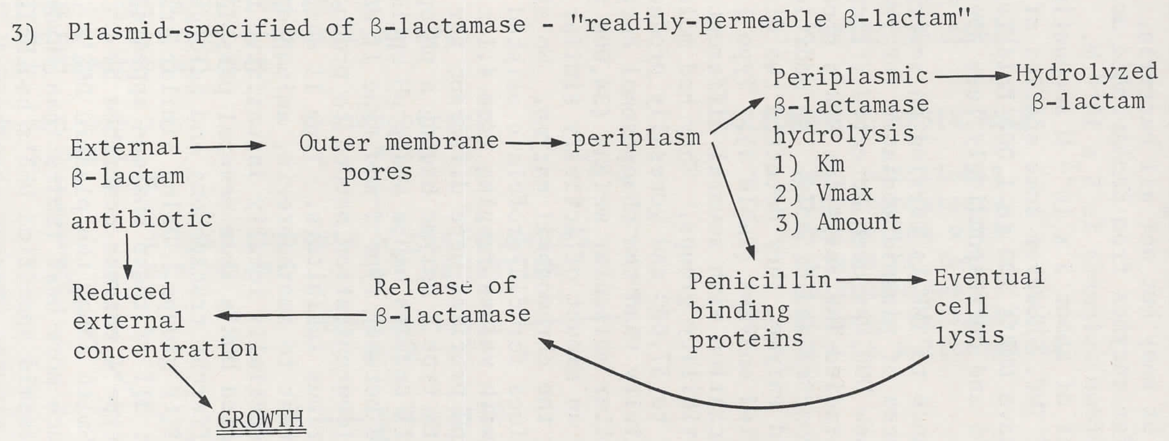

3) Plasmid-specified of β-lactamase - "readily-permeable β-lactam"
External β-lactam antibiotic
Outer membrane pores
periplasm
Periplasmic β-lactamase hydrolysis
1) Km
2) Vmax
3) Amount
Hydrolyzed β-lactam
Penicillin binding proteins
Eventual cell lysis
Release of β-lactamase
Reduced external concentration
GROWTH

Figure 2. Plasmid-specified resistance to β-lactam antibiotics in bacteria. 1) No plasmid. The route of entry of a β-lactam to target sites (penicillin-binding proteins) is shown. 2) Plasmid - with "poorly-permeable β-lactam". The entry of a β-lactam to the periplasm, contact with periplasmic β-lactamase and hydrolysis is shown. 3) Plasmid - with "readily permeable β-lactam". The entry of a β-lactam to the periplasm can exceed the rate of β-lactamase hydrolysis. Thus, some active β-lactam may reach targets, cause eventual cell lysis and release of β-lactamase. The latter hydrolyzes β-lactam external to the cell.

B. RESISTANCE TO TRIMETHOPRIM AND SULFONAMIDES

Trimethoprim (TMP) is a competitive inhibitor of dihydrofolate for dihydrofolate reductase (DHR) (Figure 3). It has a very high affinity for DHR's of many but not all bacteria. For example, the average Ki for enzymes from *Escherichia coli*, *Staphylococcus aureus* and *Proteus vulgaris* is 5 x 10^{-8} M. Mammalian DHR's have Ki values of about 3 x 10^{-3} M accounting for the selective toxicity of TMP. However some species of *Bacteroides* and *Clostridium* have DHR's 100 to 1,000 fold less sensitive than that of *E. coli* and correspondingly less sensitive to TMP (22).

Plasmid specified resistance to TMP is carried on several transposons. In those instances of plasmid mediated TMP resistance studied to date, a new DHR has been detected (Figure 3). Pattishall et al. (17) studied TMP resistant DHR's specified by R-plasmids from *Citrobacter* and *E. coli*. In addition to the chromosomally specified enzyme, they detected two distinct types of plasmid specified enzymes (Table 4). Type I enzymes were detected in association with several different plasmids of different incompatibility groups. They had molecular weights ranging from 32 to 37,000 and generally produced several times more enzyme activity than the chromosomal enzyme did. Type II enzymes had similar molecular weight (34,000 to 37,000) but normally produced an amount of activity similar to or less than that produced by the chromosomal enzyme. No significant differences in Km values for dihydrofolate existed between the two enzyme types with values ranging from 4.1 ± 0.25 µm to 11.5 ± 1 µm. The pH profiles were different for type I and type II enzymes with type I enzyme having a pH optimum about 5.5 whereas type II enzymes had a sharp pH optimum at 6.5. A striking difference between type I and type II enzymes occurred in I_{50} values (concentration needed to produce 50% inhibition of DHR) for various inhibitors. Type II enzymes were very much more resistant to amethopterin, aminopterin and pyrimethamine and were essentially totally insensitive to TMP. Amyes and Smith (1) studied DHR's from several plasmids which carried at least two different transposons and belonged to three different incompatbility groups. Using similar criteria these authors found that all of these enzymes appeared to share properties with the type I enzymes described by Pattishall et al. Amyes and Smith noted the plasmid DHR's that they studied were very much more heat labile than chromosomally specified enzymes. Plasmid specified DHR's had half lives at 45°C of 1/2 to 2-1/2 minutes whereas chromosomal enzymes had half lives of 12.2 and 15 minutes (Table 4).

Both of the studies noted above demonstrated that plasmid specified enzymes differed distinctly from the chromosomally

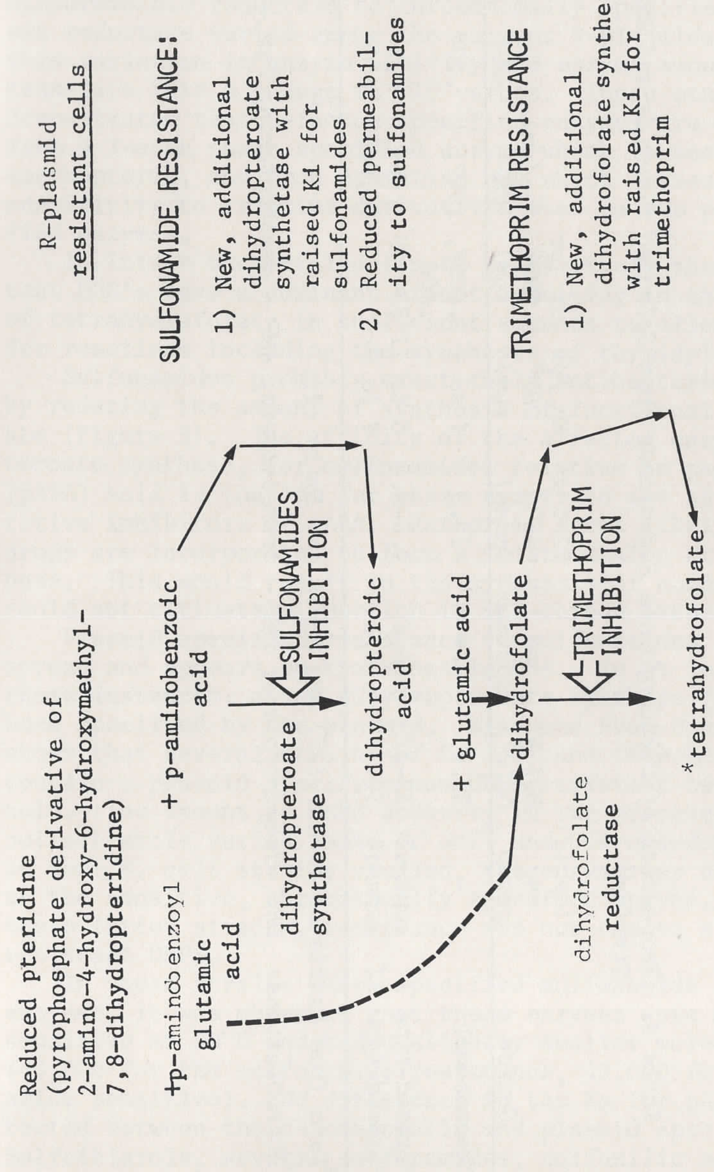

Reduced pteridine (pyrophosphate derivative of 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine)
+p-aminobenzoyl glutamic acid
+ p-aminobenzoic acid
dihydropteroate synthetase
SULFONAMIDES INHIBITION
dihydropteroic acid
+ glutamic acid
dihydrofolate
dihydrofolate reductase
TRIMETHOPRIM INHIBITION
*tetrahydrofolate
R-plasmid resistant cells
SULFONAMIDE RESISTANCE:
1) New, additional dihydropteroate synthetase with raised Ki for sulfonamides
2) Reduced permeability to sulfonamides
TRIMETHOPRIM RESISTANCE
1) New, additional dihydrofolate synthetase with raised Ki for trimethoprim

*Figure 3. Plasmid-mediated resistance to sulfonamides and trimethoprim. The normal pathway is shown on the left part of the figure. On the right is the situation with an additional plasmid-specified enzyme bypassing sites of sulfonamide or trimethoprim resistance. *1 carbon transfer - synthesis of purines, thymidylate, methionine, thiamine, pantothenate, formyl methionyl tRNA.*

Table 4. Properties of Dihydrofolate Reductases from Selected Plasmids and Bacterial Strains[a]

Source	Plasmid inc group	Mol Wt ($x10^3$)	pH optimum	T1/2 at 45°C (mins)	Km for DHF[b] (µm)	Ki for TMP[b] (µm)	I_{50} (µm) TMP	AMP[b]	Pyr[b]	Activity % NADH:NADPH
Chromosomal	-	20.5	7+	12.2	1 to 4	.0027, .006	.007,	.004	.002	8-16
Plasmid-type I	several	32-37	~5.5	1.5-2.5	5 to 11	32-150	46+	4.4+	2.5+	0
Plasmid-type II	several	34-37	6.5	-	4 to 9	-	60,000	600+	378+	-

[a] Data from Pattishal et al. (17) and Amyes and Smith (1).

[b] Abbreviations: DHF -dihydrofolate; TMP - trimethoprim; AMP - amethopterin; Pyr - pyrimethamine.

specified enzymes in several properties. Chromosomally specified enzymes were smaller (molecular weight 20,500), had a higher optimum pH (a little over 7), had much lower Ki values for TMP and much lower I_{50} values for TMP, amethopterin, aminopterin and pyrimethamine, and eluted differently from DEAE cellulose.

Amyes and Smith found that the ratio of plasmid specified dihydrofolate reductase to chromosomally specified dihydrofolate reductase varied among the various R-plasmids. However, this variation in enzyme activity and enzyme amounts did not translate into a change in MIC values. These studies have demonstrated that R-factor specified enzymes are different from a T-even phage specified enzyme which is sensitive to amethopterin, and from mammalian DHR which possesses greater sensitivity to triazine derivatives than do the plasmid specified enzymes.

In intact bacterial cells it seems likely that TMP resistant DHR's have a dominant effect resulting in the synthesis of tetrahydrofolate in sufficient amounts to allow C-1 transfer reactions including the synthesis of thymidylate.

Sulfonamides probably exert their antibacterial activity by reducing the amount of synthesis of functional dihydrofolate (Figure 3). The affinity of the affected enzyme, dihydropteroate synthase, for sulfonamides relative to p-aminobenzoic (pABA) acid is too low for these agents to act as true competitive inhibitors of pABA. Rather it seems likely that the drugs are incorporated to form a folate analog or a Schiff base. This would result in the synthesis of compounds which could not ultimately function in one-carbon transfer reactions.

Plasmid specified resistance to sulfonamides is wide spread and appears on transposable DNA. In at least some of these instances, a new dihydropteroate synthase (DHPS) has been specified by the plasmid. Wise and Abou-Donia (25) have shown that several strains of *E. coli* and *Citrobacter* which contain a plasmid specify a new DHPS resistant to sulfathiazole. The amount of DHPS activity in the presence of 10^{-4} M sulfathiazole varied among *E. coli* and *Citrobacter* strains. In four *E. coli* strains studied, the enzyme was as sensitive as the sensitive, chromosomally specified enzyme. Thus in these latter strains, resistance was not due to a sulfonamide resistant DHPS.

In those strains which specified sulfonamide resistant enzymes, it was observed that these enzymes were more heat sensitive at 50°C and had a slightly smaller molecular weight (45,000 for the sulfonamide resistance, 49,000 for the sulfonamide sensitive). No difference in the Km for pABA was detected between the chromosomally and plasmid specified enzymes. Sulfathiazole, several sulfonamides, sulfanilic acid and arsanilic acid inhibited the sensitive strain but had little effect on resistant enzymes. However small pABA analogues such as p-aminosalicylic acid had similar I_{50} values for both sulfonamide sensitive and resistant enzymes. These studies examined highly sulfonamide resistant strains and did not look at strains with lower level resistance (less than a 1,000 micrograms/ml).

Previous studies have shown that some strains of bacteria have shown a reduction in permeability for sulfonamides. However the reduction in permeability was small relative to the increase in resistance. This possible mechanism of resistance requires further study. It has also been shown that some strains of *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Neisseria gonorrhoea* apparently have an increase in pABA (13, 23). It is possible this increase would overcome the inhibitory effect of sulfonamides.

It has also been observed that some strains of bacteria which are resistant to the trimethoprim-sulfamethoxazole combination require thymine or thymidine. In these instances it is likely that there is a reduced requirement for tetrahydrofolate in the C-1 transfer reaction to synthesize thymidylate.

C. MECHANISMS OF PLASMID-MEDIATED AMINOGLYCOSIDE RESISTANCE

Plasmid mediated resistance to aminoglycoside antibiotics is associated either with enzymic modification of aminoglycosides or with apparent impermeability to these drugs in the absence of enzymic modification. The latter mechanism has not been detected as a widely distributed form of resistance (11). However apparent impermeability not associated with a plasmid has been widely described particularly amongst strains of *Pseudomonas aeruginosa* (2).

A large number of different enzymes exist which are capable of N-acetylation or 0-phosphorylation or 0-nucleotidylation of various aminoglycosides (7) (Table 5). Enzymes are

Table 5. Plasmid-Specified Mechanisms of Aminoglycoside Resistance

A) Enzymic modification

1) N-acetylation - 6', 2' or 3-amino group
2) 0-phosphorylation - 3", 3', 5", 2" or 6 hydroxyl group
3) 0-nucleotidylation - 3", 2" 4' or hydroxyl group

B) Apparent impermeability

Table 6. Enzymic Modification of Aminoglycosides

A) Phosphorylation	
APH (6)	*6-hydroxyl of streptidine ring - Streptomycin*
APH (3")	*3" - hydroxyl of glycosamine ring (III) Streptomycin*
APH (3')-I	*3' - hydroxyl of aminohexose I of some deoxystreptamine aminoglycosides*
-II	*Same as APH(3')-I but different substrate range*
-III	*Same as APH(3')-I but different substrate range*
APH (5")	*5"-hydroxyl of ribose of ribostamycin*
APH (2")	*2"-hydroxyl of aminohexose III of some deoxystreptamine aminoglycosides*
B) Acetylation	
AAC (6')	*Several subtypes; 6'-amino of aminohexose I of some deoxystreptamine aminoglycosides*
AAC (2')	*2'-amino of aminohexose I of some deoxystreptamine aminoglycosides*
AAC (3)-I	*3-amino group of deoxystreptamine ring*
AAC (3)-II	*Same as AAC(3)-I but different substrate range*
-III	*Same as AAC(3)-I but different substrate range*
-IV	*Same as AAC(3)-I but different substrate range*
C) Nucleotidylation	
AAD (3") (9)	*3"-hydroxyl on aminohexose III of streptomycin or 9-hydroxyl of spectinomycin*
ANT (2")	*2"-hydroxyl of aminohexose III of some deoxystreptomine aminoglycosides*
ANT (4') or (AAD(4')	*4'-hydroxyl of aminohexose I of some deoxystreptamine aminoglycosides*
AAD (6)	*6-hydroxyl of streptidine ring of streptomycin*

APH - aminoglycoside phosphotransferase; AAC - aminoglycoside acetyltransferase; AAD - aminoglycoside adenylyltransferase; ANT - aminoglycoside nucleotidyltransferase.

classified by the nature of the transfer reaction which occurs and by the site of the aminoglycoside molecule on which modification takes place. For example, an enzyme capable of acetylating the 6' amino group of certain aminoglycosides is termed aminoglycoside 6' acetyl transferase AAC(6'). However, not all enzymes which fall into such groups are identical. One major way in which some enzymes vary is by the profile of substrates which are modified. Thus the enzyme AAC(6')-I acetylates a somewhat different group of aminoglycosides than does AAC(6')-II. The naming of the subtypes of major enzymic groups is not complete nor in all instances totally agreed upon. However, several subtypes are currently recognized and are listed in Table 6. In order to achieve a more satisfactory nomenclature for the subtypes or subclasses it will be necessary to determine substrate profiles under highly defined conditions and to use enzymes of defined purity.

In spite of the obvious and generally excellent correlation between resistance and the presence of enzymes capable of modifying aminoglycosides, resistance is not simply due to inactivation of the antibiotic. A variety of investigators have made this conclusion based on several observations (Table 7). When a strain acquires a plasmid specifying enzymic modification of the aminoglycoside, it is possible to detect only very minute quantities of modified or inactivated aminoglycoside in the extracellular medium of growing cells, thus excluding detoxification of the medium as the resistance mechanism. In addition, it has been demonstrated that at least some of the aminoglycoside modifying enzymes are located on the exterior surface of the cytoplasmic membrane and, perhaps to some extent, also in the periplasmic space.

Table 7. Evidence that Plasmid-Specified Aminoglycoside Resistance Associated with Enzymic Modification Results from Modification of Drug in the Process of Transport

1) *Modification results in inactive aminoglycosides.*
2) *The external concentration of aminoglycoside is reduced less than 1% by growing R^+ cells.*
3) *A clear correlation exists between the amount of enzyme activity, the amount of drug transported and the level of resistance.*
4) *Enzymic activity is present on the outer surface of the cytoplasmic membrane.*
5) *Modified drug can be recovered from bacterial cells and increases in amount with time.*

We have observed for sometime that the possession of R-plasmid specified aminoglycoside modifying enzyme results in a reduction in the amount of aminoglycoside accumulated with time. We have been able to show this for enzymes capable of acetylating and adenylyating gentamicins and phosphorylating and adenylating streptomycin. These findings plus the observations that enzymically modified aminoglycosides have reduced affinity for ribosomal binding sites have led to a concept of how aminoglycoside resistance is achieved in strains possessing aminoglycoside modifying enzymes (3,8). To date, most investigations have been carried out on *E. coli* and other Gram-negative bacteria. However some information is also available on Gram-positive bacteria. (For references see 8,7).

In Gram-negative bacteria it appears likely that aminoglycosides penetrate through polysaccharide extracellular layers and through the polysaccharide component of lipopolysaccharide by diffusion (Figure 4). As most of these polysaccharides carry a generally negative charge it is probable that there is some binding of aminoglycosides to the polysaccharide. As a result, in some strains of certain bacteria these layers may act as a minor penetration barrier. It is possible, for example, in very mucoid strains of *Pseudomonas aeruginosa* that the mucoid extracellular layer retards the entry of aminoglycoside to a relatively minor degree. Following penetration of the outer layers, aminoglycosides diffuse through pores that exist in the outer membrane of Gram-negative bacteria and reach the peptidoglycan. The peptidoglycan itself is likely a minor barrier to aminoglycoside entry but disruption of the peptidoglycan can result in extensive disruption of the outer membrane and reduce the barrier posed by that structure. Having penetrated the cell wall component, aminoglycosides enter the periplasmic space and bind to polar heads of phospholipids of the cytoplasmic membrane. Here they come in contact with the modifying enzymes and undergo modification prior to crossing the cytoplasmic membrane to reach the ribosome (Figure 4).

Recent studies performed in our laboratory have shown that modified aminoglycosides are accumulated with kinetics which are identical to those seen in the first phase of energy dependent accumulation of unmodified aminoglycosides by bacteria. It is also clear from studies using bacterial mutants with reduced enzymic activity and mutants which have reduced transport capabilities that there is a competition between the rate of inactivation and the rate of transport (Figure 5) (8). This allows an explanation of the observation that possession of an enzyme able to modify aminoglycoside does not always result in resistance to that drug. If the Km for the aminoglycoside in question were significantly higher than the concentration necessary to achieve transport to the ribosome,

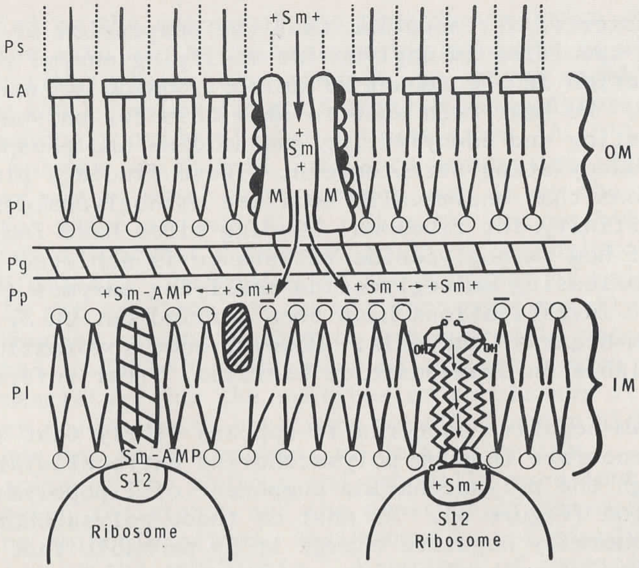
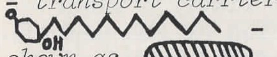


Figure 4. Penetration of streptomycin or gentamicin through the cell envelope of the Gram-negative bacterium with an R-factor specified enzyme (left section) or no R-factor (right section). +Sm+, positively charged streptomycin or gentamicin; $\bar{o}$, negatively charged polar head of phospholipid; +Sm-AMP, adenylylated streptomycin. Alternative possible transport sites for Sm or gentamicin are shown as: ▨ *- transport carrier of a structurally related compound;*  *- respiratory quinone. Modifying enzyme is shown as* ▨ *; M = Matrix protein; OM = Outer membrane; IM = Inner membrane; Ps = polysaccharide; LA = Lipid A; Pl = Phosolipid; Pg = Peptidoglycan; Pp = periplasm.*

significant enzymic modification would not result prior to ribosomal binding of the active aminoglycoside. The last situation would result in a failure to express resistance in spite of an enzyme modifying an aminoglycoside.

When enzymically modified aminoglycoside is transported across the cytoplasmic membrane, it fails to bind to the ribosome. As a result the sequelae of ribosomal binding of the aminoglycosides do not occur and the strain is resistant to the drug as long as the rate of inactivation exceeds the rate of transport. This appears to be an efficient mechanism of producing resistance as it results in a requirement only to inactivate a very small fraction of aminoglycoside present. Only that fraction of the drug which is actually undergoing transport needs to be inactivated.

Growth or inhibition of growth of bacteria at a given concentration of an aminoglycoside is determined by:

Rate of Enzymic Modification (Mrate) vs Rate of Transport (Trate)

- function of:	- function of:
Km	energy source*
Vmax	periplasmic or extracellular concentration
Amount per cell	

If Mrate > Trate ⟶ Growth

Mrate < Trate ⟶ Inhibition

* energy source - Electron transport (E.T.) to oxygen is most effective whereas E.T. to nitrate is less effective. Least effective is E.T. to fumarate or ATP from substrate phosphorylations. The major driving force is probably the electronegativity component of the cellular proton motive force (PMF). It is enhanced for example by an increase in pH reducing the pH differential between the inside and outside of the cell, thus increasing internal electronegativity to maintain the cellular PMF.

Figure 5. "Rate-Competition" explanation of aminoglycoside resistance due to R-specified enzymic modification. Mrate - rate of enzymic modification; Trate - rate of transport.

The process of energy-dependent aminoglycoside transport is known to involve biphasic accumulation kinetics. The last phase (energy dependent phase II) is dependent on ribosomal binding of the aminoglycoside and is initiated by interaction with ribosomes. The first energy dependent (EDP-I) accumulation phase represents initial transport of these drugs across the cytoplasmic membrane. Two observations concerning the rate of EDP-I uptake are essential to understand enzymic resistance. First, EDP-I is a very slow rate and it is this phase of transport with which enzymic inactivation must compete. This explains why only a small fraction of all drug need be inactivated. Secondly, the rate of transport is directly concentration dependent; at high concentrations it can exceed the rate of inactivation.

The exact mechanism by which initial transport (EDP-I) occurs is not finally established. However it is an energy dependent process with the most effective source of energy being supplied by terminal electron transport to oxygen. Terminal electron transport to nitrate is considerably less effective but much more effective than terminal electron transport to fumarate or an energy source such as glucose which supplies ATP through substrate phosphorylations. Energy-dependent entry requires a proton gradient as proton-leaky bacterial mutants and uncoupling agents stop or reduce accumulation. However, in view of the cationic nature of aminoglycosides and their strong dependence for activity on pH, it is likely that the driving force for entry is the magnitude of the internal electro-negativity component of the cellular proton motive force (Bryan, L.E., manuscript in preparation).

It is possible that carriers for other compounds sharing structural similarities are used by aminoglycosides to cross the cytoplasmic membrane. However an alternative explanation is that the drug binds to some component of the cytoplasmic membrane involved in proton translocation across the cytoplasmic membrane. A possible candidate would be respiratory quinones (Figure 4).

It is probable that plasmid mediated aminoglycoside modification and resistance is achieved in a similar manner in Gram-positive bacteria. Our own studies using *Staphylococcus aureus* have shown that less than 1% of extracellular gentamicin is modified and there is a reduction in gentamicin accumulation in a strain possessing plasmid-specified gentamicin modifying enzymes compared to the gentamicin accumulation by an isogenic enzyme-negative strain.

The results are similar but not identical to the pattern seen with Gram-negative bacteria, suggesting the mechanisms of resistance may be similar.

D. CHLORAMPHENICOL RESISTANCE

The most widely described form of plasmid-specified chloramphenicol resistance has been associated with the presence of a chloramphenicol acetyltransferase. Chloramphenicol resistance has been shown to be carried by transposon 9. This transposon differs from other transposable resistance in that it possesses a direct repeat and not an inverted repeat sequence. As well the frequency and sites of transposition appear more limited than for other transposons.

Chloramphenicol acetyltransferases have been found in a wide variety of bacteria and in many instances are plasmid-specified. Fitton et al. (9) have recently summarized many of the properties of these enzymes including some information on their amino acid sequences. The most wide spread chloramphenicol acetyltransferase is the type I specified by the Tn9 transposon and often carried by F-like plasmids. Enzymes have been classified on the basis of plasmid linkage, mode of synthesis (constitutive or inducible), subunit molecular weight, native electrophoretic mobility, thiol reagent sensitivity, affinity chromatography binding, and immunological class. Interestingly there is a high degree of identity of amino acid sequence of the active centre peptides from monomers of both Gram-negative and Gram-positive bacterial enzymes.

The possession of a chloramphenicol acetyltransferase is usually associated with extensive detoxification of the medium. However this varies depending on the type of bacteria possessing the enzyme. Although many strains of *Staphylococcus aureus* and *E. coli* possessing these enzymes do detoxify the medium, many strains of *Pseudomonas aeruginosa* do not (16). The mechanism of resistance for strains which failed to detoxify the medium in association with chloramphenicol acetyltransferase activity is unknown.

Resistance to chloramphenicol has also been described in the absence of chloramphenicol acetyltransferase activity (16, 11). Evidence supplied to date is consistent with a reduced permeability to chloramphenicol in these types of strains. However, the mechanism for reduction in chloramphenicol accumulation is not known.

E. RESISTANCE TO HEAVY METALS

Plasmid-specified resistance to heavy metals has recently been reviewed by Summers and Silver (19). Only resistance to mercury and cadmium will be briefly examined in this report.

Resistance to mercury has been shown to be associated with reduction of Hg^{2+} to flavoproteins and to require a thiol reagent for activity. NADPH seems to be the preferred electron donor. The Hg^0 is volatile and is lost from the medium. Resistance to organomercurcials involves one or more lyase enzymes responsible for removing the organic component and releasing Hg^{2+}.

In all example of mercury resistance examined to date, resistance is inducible. Resistance tends to appear in a narrow and broad spectrum resistance pattern. For example, in *E. coli* the plasmids which confer resistance to inorganic Hg^{2+} also confer resistance to the organomercurials merbromin and fluoroscein mercuric acetate. Plasmids showing broad spectrum resistance express resistance to the preceding agents as well as phenylmercuric acetate and thimersal. Plasmids from *Pseudomonas aeruginosa* also show a broad and narrow spectrum of resistance although the patterns are slightly different. Strains of *Staphylococcus aureus* so far express only the broad spectrum of resistance without showing resistance to merbromin.

The mercury reductase enzymes are intracellular in location. Recent work has shown that a transport system for Hg^{2+} is also specified by a plasmid mediating Hg^{2+} resistance (Foster, T., personal communication).

In the case of certain mercury derivatives, there is no evidence of detoxification. For example, merbromin and fluorescein mercuric acetate are not apparently detoxified. This and other observations have suggested that in some instances mercuric resistance may be produced through an alteration of permeability to the compounds.

Weiss et al. (24) have recently shown that a strain of *Staphylococcus aureus* containing a plasmid expresses resistance through a reduction in an accumulation of cadmium. It is likely cadmium enters staphylococcal cells by an energy dependent manganese transport system. In the presence of the plasmids this system no longer accepts cadmium as an alternative substrate (Figure 6).

F. RESISTANCE TO OTHER ANTIBIOTICS

As noted earlier (Table 1), resistance to several other antibiotics is specified by plasmids. Owing to space and time limitations it is not my intention to review resistance to other agents in detail. Chopra and Howe (5) have recently reviewed resistance to tetracycline. In general it has been recognized for some time that resistance to tetracycline is associated with a reduced accumulation of tetracycline. However, it is not yet known whether this is due to a reduced

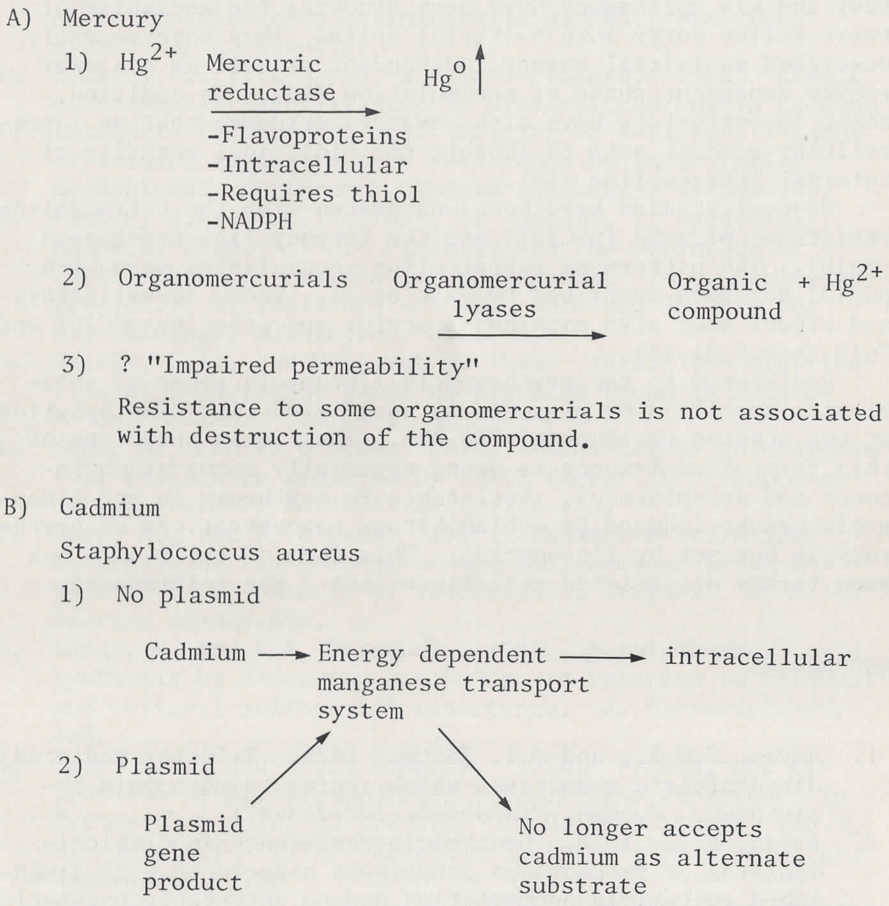

A) Mercury
1) Hg2+
Mercuric reductase
Hg0
-Flavoproteins
-Intracellular
-Requires thiol
-NADPH
2) Organomercurials
Organomercurial lyases
Organic compound
+ Hg2+
3) ? "Impaired permeability"
Resistance to some organomercurials is not associated with destruction of the compound.
B) Cadmium
Staphylococcus aureus
1) No plasmid
Cadmium
Energy dependent manganese transport system
intracellular
2) Plasmid
Plasmid gene product
No longer accepts cadmium as alternate substrate

Figure 6. Plasmid-specified resistance to mercury and cadmium. Hg^{2+} - inorganic mercuric ion; Hg^0 - mercury; ↑ - volatile.

rate of entry or an increased rate of efflux of the antibiotic. Levy and his colleagues have been studying the mechanism of tetracycline entry into bacterial cells. They have recently described an initial energy independent as well as a slower energy dependent phase of accumulation (15). In addition, these investigators have also advanced evidence that an intracellular product acts to inhibit the biological activity of internal tetracycline (14).

Several studies have been undertaken with the tetracycline resistance plasmid (pSC101) and the tetracycline transposon (Tn10). The pattern of tetracycline accumulation seen with pSC101 has been described by Tait et al. These investigators and others have also examined proteins specified by pSC101 and Tn10 (see 5,21,14).

Resistance to the erythromycin-lincomycin group of antibiotics specified by plasmids is associated with dimethylation at two adenine residues of 23S RNA. The common phenotype of this form of resistance is found especially among staphylococci and streptococci. Resistance to erythromycin and lincomycin can be induced by subinhibitory concentrations of erythromycin but not by lincomycins. This form of resistance has been termed dissociated resistance (see 7 for references).

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DISCUSSION

Foster: I'd like to make one comment on Hg resistance: in collaboration with Simon Silver, we have evidence to suggest that plasmids which specify mercuric reductase also specify an inducible mercuric ion transport system. If you make mutations which abolish reductase activity the cells become hypersensitive to mercuric ions. This hypersensitivity correlates with hyper binding of radioactive mercuric ions to the cell; the Hg^{2+} hyper binding is inducible by subtoxic levels of Hg^{2+}. We think that the transport and reductase genes form an inducible operon, with the reductase gene being the more distal.

Behme: I have two questions: firstly, are streptomycin-dependent *E. coli* obligate aerobes?, and secondly, with regard to transport of streptomycin in sensitive cells, I wonder if you've correlated that with cell death; I know that radiation-injured *E. coli* and *Serratia* take up streptomycin very rapidly.

BRYAN: First of all, streptomycin-dependent cells are nearly obligate aerobes. The interesting thing is that you can make them grow very slowly if you have nitrate reductase activity which is the other major energetic source for aminoglycoside transport. Fumerate reductase is relatively ineffective and substrate phosphorylation by ATP is also relatively ineffective. But they don't grow very well in the presence of nitrates. They do grow, but very slowly, so for practical purposes they almost are strict aerobes - the ones I've looked at I should say. In terms of transport, what is necessary is a functioning electron transport system, you don't have to have a living cell. I don't know how to define death in bacteria. It's true that when they swing into the accelerated phase of transport those organisms are basically committed to death at that point and subsequently, in a few minutes, they will start to leak intracellular components. But the transport system is always energy dependent.

Shapiro: In the model of where the modifying enzymes are in the cytoplasmic membrane, don't they actually have to span the membrane? They use acetyl-CoA or ATP as cofactors and those are on the inside of the membrane. Also, all of the known integral membrane proteins are exposed on both sides of the membrane.

BRYAN: Yes, you're probably right on that, I 've just taken artistic license in that drawing.

PLASMID DETERMINED RESISTANCE TO CARBENICILLIN AND GENTAMICIN IN *Pseudomonas aeruginosa*[1]

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Pseudomonas aeruginosa remains an important opportunistic pathogen in hospitalized patients despite the introduction of effective anti-pseudomonas antibiotics such as carbenicillin and gentamicin. One reason for this continuing problem is the appearance of *P. aeruginosa* isolates resistant to these drugs in many hospitals (6,7). For example, Table 1 documents the emergence of resistance in *P. aeruginosa*, as contrasted to *Escherichia coli*, in clinical isolates at the Massachusetts General Hospital since gentamicin was introduced in 1971. Currently 19% of *P. aeruginosa* isolates are resistant to gentamicin by disc susceptibility testing.

R plasmids that code for antibiotic inactivating or modifying enzymes contribute to this resistance, although other mechanisms also occur. Transmissible R plasmids have been found in *P. aeruginosa* strains from many countries (reviewed in 16) and are estimated to be present in less than ten percent of nonselected strains (20,21,33). Many are not transmissible by conjugation to *E. coli* or other enterobacteria so that a *Pseudomonas* recipient must be used for their detection (2). Fertility plasmids are even more common than R plasmids (4) and cryptic plasmids have also been detected by physical techniques (16). Several procedures for agarose gel electrophoresis allow *Pseudomonas* plasmids to be visualized and given provisional molecular weights (5,9,24,27). By incompatibility tests plasmids found in *P. aeruginosa* can also be classified into eleven or more incompatibility (Inc) groups (14,15,19,24, 29). Recently the enzymes responsible for plasmid-determined

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Table 1. Percent Gentamicin Resistant Isolates at Massachusetts General Hospital

	Year						
Organism	*1971*	*1973*	*1974*	*1975*	*1976*	*1977*	*1978*
E. coli	*1*	*1*	*1*	*1*	*2*	*2*	*2*
P. aeruginosa	*1*	*5*	*8*	*9*	*14*	*15*	*19*

carbenicillin resistance have been characterized and a group of gentamicin resistant *P. aeruginosa* isolates has been examined in detail to determine more about the molecular mechanisms of resistance.

PLASMID DETERMINED RESISTANCE TO β-LACTAM ANTIBIOTICS

The β-lactamases determined by plasmids of Gram negative bacteria can be divided into at least eleven types by biophysical and biochemical criteria (25,26,32). Four of these enzymes have been termed pseudomonas-specific enzymes (PSE) since they have not been found in other genera despite extensive search (11). Differences in isoelectric point allow the enzymes to be distinguished conveniently by isoelectric focusing. Differences in substrate profile and susceptibility to inhibitors also allow all but the TEM-1 and TEM-2 types to be differentiated.

Thirty-four plasmids that were found in *P. aeruginosa* and that determine resistance to carbenicillin have been examined by isoelectric focusing for the type of β-lactamase produced (15, A.A. Medeiros and G.A. Jacoby, unpublished data). Nine of the eleven enzyme types have been found. Some of their distinguishing properties are listed in Table 2. The OXA enzymes are so termed because of their relatively high activity toward oxacillin and other isoxazoyl β-lactam substrates, and SHV-1 is so named because inhibition by sulfhydryl reagents varies according to the substrate employed (26). The distribution of β-lactamases encoded by different pseudomonas plasmids listed by their incompatibility groups is given in Table 3. As with plasmids of other Gram negative organisms TEM-type enzymes were the most common. Eight plasmids produced a TEM-1 enzymes and 12 plasmids made TEM-2 β-lactamase. The plasmids involved belong to five different Inc groups, a finding consistent with the known determination of TEM-1 and TEM-2 by transposable sequences of DNA (10,12). The OXA-2 and OXA-3 β-lactamases were the next most common and were also

Table 2. Properties of β-lactamases Specified by Plasmids Found in P. aeruginosa[a]

	Relative rate of hydrolysis					
β-lactamase	*Penicillin G*	*Carbenicillin*	*Oxacillin*	*Cephaloridine*	*Molecular Weight ($x10^3$)*	*Isoelectric point*
TEM-1	*100*	*10*	*5*	*76*	*22.0*	*5.4*
TEM-2	*100*	*10*	*5*	*74*	*23.5*	*5.6*
SHV-1	*100*	*8*	*0*	*56*	*17.0*	*7.6*
OXA-2	*100*	*15*	*646*	*37*	*44.6*	*7.45,7.7*
OXA-3	*100*	*10*	*336*	*44*	*41.2*	*7.1*
PSE-1	*100*	*97*	*<2*	*18*	*28.5*	*5.7*
PSE-2	*100*	*121*	*317*	*32*	*12.4*	*6.1*
PSE-3	*100*	*253*		*10*	*12.0*	*6.9*
PSE-4	*100*	*150*	*8*	*40*	*32.0*	*5.3*

[a] *Data from 15,25,26.*

Table 3. Distribution of β-lactamase Genes on Pseudomonas Plasmids

β-Lactamase	*Plasmid*	*Incompatibility group*
TEM-1	*R527, R1033*	*P-1*
	Rm16a, pMG20, pMG21, Rms165	*P-2*
	R2, pMG18	*P-9*
TEM-2	*RP1, RP4, Rm16b, R30, R68, RP638*	*P-1*
	pMG23, R40c, R91	*P-10*
	RP1-1, RP8, RP56Be	*P-11*
OXA-2	*pMG40, pMG44*	*P-2*
	pMG35, pMG48	*unclassified*
OXA-3	*RIP64*	*P-3*
	pMG25, pMG26	*unclassified*
SHV-1	*pMG22*	*P-1*
PSE-1	*RPL11, Rms139*	*P-2*
PSE-2	*pMG33, Rms151*	*unclassified*
PSE-3	*Rms149*	*P-6*
PSE-4	*pMG19*	*unclassified*

produced by plasmids of several Inc groups. Whether these enzymes are determined by transposons is not yet known. SHV-1 was determined by a single plasmid. The gene for this enzyme has recently been found to be transposable (28). The genes for PSE-type enzymes occur on plasmids that are either intrinsically transfer deficient (Tra⁻), such as pMG19 and Tms149, or on plasmids whose host range is restricted to *Pseudomonas*. In the laboratory PSE-type β-lactamase genes can be transmitted on plasmids to *E. coli* where they function efficiently (11). It may be only a matter of time until one of the PSE-type β-lactamase genes is found on a plasmid occurring naturally in enterobacteria.

The emergence of carbenicillin resistant *P. aeruginosa* has spurred the development of newer β-lactam antibiotics that are more potent toward *Pseudomonas* and more resistant to β-lactamase hydrolysis. One of the most promising new drugs is cefotaxime (HR756), a cephalosporin derivative with a broad spectrum of activity against Gram positive and Gram negative organisms. As one predictor of clinical effectiveness, the activity of cefotaxime has been compared with that of carbenicillin against *Pseudomonas* strains producing each of the β-lactamase types (18 and unpublished data). Table 4 shows that, in contrast to carbenicillin, the minimum inhibitory concentration of cefotaxime is unaffected by plasmid-determined mechanisms of β-lactam resistance.

Table 4. Plasmid-Determined Resistance to Carbenicillin and Cefotaxime

		Minimum inhibitory concentration (μg/ml)	
β-Lactamase	*Plasmid*	*Carbenicillin*	*Cefotaxime*
	none	25	20
TEM-1	*R527*	2000	20
TEM-2	*RP1*	8000	20
SHV-1	*pMG22*	400	20
OXA-2	*pMG35*	800	20
OXA-3	*RIP64*	2000	20
PSE-1	*RPL11*	4000	20
PSE-2	*R151*	200	20
PSE-3	*Rms149*	4000	20
PSE-4	*pMG19*	8000	20

PLASMID-DETERMINED RESISTANCE TO GENTAMICIN AND OTHER AMINOGLYCOSIDES

Pseudomonas plasmids are known to make enzymes capable of acetylating or adenylylating gentamicin and other aminoglycosides (3). Both 3- and 6'-acetyltransferases have been described, and each can be subdivided into several types based on substrate range. Phosphotransferases active against kanamycin and related antibiotics also occur as well as clinical strains resistant to many aminoglycosides that appear not to modify or inactivate the drugs but rather to possess a permeability block that prevents their access to the ribosome (1). A few *Pseudomonas* plasmids have even been described that may determine resistance to streptomycin or kanamycin by impermeability (22,23).

To gain a better understanding of the variety of mechanisms used to achieve aminoglycoside resistance we have recently begun the analysis of fifty gentamicin resistant clinical *P. aeruginosa* isolates collected from hospitals throughout the United States and supplied by George Miller of the Schering Corporation. By enzymatic assay (8) with gentamicin as substrate twenty of the strains made aminoglycoside acetyltransferase (AAC), ten strains determined aminoglycoside adenylyltransferase (AAD), and for the remaining twenty strains neither activity could be measured. It should be emphasized that low levels of aminoglycoside modification could easily have escaped detection so that the enzyme negative category is provisional. These 50 strains have been examined for plasmids by techniques of agarose gel electrophoresis that allow plasmids from a few to over 300 x 10^6 in molecular weight to be visualized (5,9,27). The strains have also been tested for transmissibility of resistance to gentamicin, and other plasmid-determined resistances, using at least one *P. aeruginosa* recipient. The results are summarized in Table 5.

Extrachromosomal DNA was seen in 32 of these 50 strains, but only 14 strains, including two in which plasmid DNA had not been visualized, transferred resistance to gentamicin on mating. The high frequency of non-donor strains was unexpected. The resistance properties of nine non-donor strains are shown in Table 6. These isolates all made aminoglycoside modifying enzymes and were resistant to multiple antibiotics as well as to compounds of boron, mercury, and tellurium. Resistance to these compounds is known to be plasmid-determined in *Pseudomonas sp.* (30,31). Some contained one or more plasmids, but in others plasmids were not visualized even by techniques that allow large and fragile IncP-2 plasmids to be seen (9). A certain humility about physical detection of plasmids in

Table 5. Plasmids and Aminoglycoside Modifying Enzymes in 50 Gentamicin Resistant P. aeruginosa *Clinical Isolates*

	AAC^+	AAD^+	"AAC^- AAD^-"
R plasmids detected by conjugation, mobilization, or transformation	5	5	7
Plasmid$^+$ but no antibiotic resistance transfer	9 (2 FP^+)	2	6 (2 FP^+)
Plasmid$^-$ and no antibiotic resistance transfer	6 (1 FP^+)	3 (1 FP^+)	7
Total gentamicin resistant clinical isolates	20	10	20

Pseudomonas sp. is advisable. Some well studied pseudomonas plasmids still cannot be seen by physical techniques (13), and in this study plasmids were found by mating experiments in two strains in which they had not been seen on electrophoresis. The plasmid-negative strains unable to donate gentamicin resistance could contain such nonvisualizable R plasmids that are also transfer deficient (Tra$^-$). Alternatively, resistance could be determined by chromosomal genes or chomosomally integrated plasmids. Experiments to distinguish among these possibilities are in progress.

Table 6. Properties of Selected Non-Donor P. aeruginosa *Strains*

Strain	*Origin*	*Resistance phenotype*	*Plasmids visualized (mol. wt. x 10^6)*
SCH6	*Ohio*	*Gm (AAC^+) Km Sm Su Bor Hg Ter*	*none*
SCH11	*California*	*Gm (AAD^+) Km Sm Su Tc Tm*	*none*
SCH31	*New York*	*Gm (AAC^+) Km Sm Su Bor Hg Ter*	*none*
SCH42	*Illinois*	*Cb Gm (AAD^+) Sm Su Tc Tm Hg Pmr Ter*	*none*
SCH67	*New York*	*Gm (AAC^+) Km Sm Tm Hg*	*none*
SCH27	*Massachusetts*	*Cm Gm (AAC^+) Km Sm Tm Bor Hg Pmr*	*21, 68*
SCH61	*Pennsylvania*	*Gm (AAC^+) Km Sm Tm Hg*	*30*
SCH68	*New York*	*Gm (AAD^+) Km Sm Su Tc Tm Bor Hg Pmr*	*58*
SCH69	*New York*	*Cb Cm Gm (AAC^+) Km Sm Tc Tm Bor Hg Ter*	*3.5, 55, 83*

The non-donor strains carrying plasmids could also contain Tra$^-$ R plasmids or fertility plasmids. Many have been tested for chromosome donor ability and for transmissible mercury resistance. Six fertility plasmids were found, of which four, like the FP2 pseudomonas fertility plasmid, carried resistance to mercury ions and phenylmercuric acetate. Tra$^-$ plasmids may be present in the remaining strains. In fact, when the fourteen recipients that had received gentamicin resistance by mating were tested for serial transmissibility of resistance, two of the transconjugants were not able to retransfer resistance to a further recipient and hence apparently carried Tra$^-$ plasmids. A coexisting Tra$^+$ plasmid in the original isolate may have allowed the Tra$^-$ plasmid to be mobilized or a coexisting bacteriophage may have allowed it to be transduced to the original recipient. By introducing Tra$^+$ plasmid R751 into non-donor strains carrying other plasmids, one additional Tra$^-$ plasmid has been detected by mobilization, and by utilizing transformation with plasmid DNA purified from non-donor strains two more Tra$^-$ plasmids have been found. Additional studies may reveal that Tra$^-$ plasmids are as common as Tra$^+$ plasmids in this collection. Already it is evident that estimates of R plasmid prevalence in *P. aeruginosa* that rely solely on mating techniques are likely to underestimate true plasmid carriage.

Some of the properties of the 17 new R plasmids detected in this study are listed in Table 7. Five of the plasmids belong to IncP-2, previously found to be the commonest group of classifiable R plasmids in *Pseudomonas sp.* (29). The plasmids in this group characteristically determine resistance to tellurium compounds (30) as well as to multiple antibiotics and all have molecular weights near 300 million. One plasmid each belonged to Inc groups P-9, P-10, and P-11. In particular, IncP-11 plasmid pMG39 has been very valuable in defining this group since it is the first P-11 plasmid to determine resistance to more than carbenicillin. Nine of the plasmids have not yet been classified, in most cases because they have proven compatible with representatives of all the Inc groups against which they could be tested. Further studies will very likely lead to the definition of additional Inc groups. Five unclassified plasmids are Tra$^-$, and compatibility tests are planned using transformation to test them against each other and additional known Tra$^-$ pseudomonas plasmids. Interestingly, two of the Tra$^-$ plasmids, both from strains isolated in Chicago, determine the same DNA restriction and modification

Table 7. Properties of R Plasmids Detected by Genetic Tests

Inc group	*Plasmid*	*Origin*	*Resistance phenotype*	*Estimated molecular weight x* 10^6
P-2	*pMG31*	*Louisiana*	*Gm Su Tc Tm Hg Pmr Ter Uv*	*300*^{a}
	pMG37	*California*	*Gm Km Sm Su Tm Hg Pmr Ter Uv*	*300*
	pMG40	*Illinois*	*Cb Gm Km Su Tm Hg Ter Uv*	*300*
	pMG43	*Ohio*	*Gm Sm Su Bor Hg Pmr Ter*	*300*
	pMG44	*Illinois*	*Cb Gm Km Su Tm Hg Ter Uv*	*300*
P-9	*pMG38*	*Maryland*	*Gm Km Su Tc Tm Hg Uv*	*b*
P-10	*pMG41*	*New York*	*Gm Km Sm Su Tm Hg Pmr*	*63*
P-11	*pMG39*	*Illinois*	*Cb Gm Km Sm Tm*	*57*
Unclassified	*pMG25*		*Cb Cm Gm Km Sm Su Tm Bor*	*b*
	pMG26		*Cb Cm Gm Km Sm Su Tm Bor*	*b*
	pMG29	*Texas*	*Gm Sm Su Tm Hg*	*110*
	pMG30	*Texas*	*Gm Sm Su Tm Hg*	*110*
	pMG32	*Illinois*	*Gm Km Sm Su Tm PaeR7* Tra^-	*18*
	pMG33	*Illinois*	*Cb Gm Km Sm Su Tm PaeR7* Tra^-	*30*
	pMG35	*Delaware*	*Cb Gm Km Sm Su Tm Hg Pmr* Tra^-	*14*
	pMG48	*Delaware*	*Cb Gm Km Sm Su Tm Hg Pmr* Tra^-	*16*
	pMG49	*California*	*Gm Sm Su Hg Pmr* Tra^-	*20*

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[a] *Estimate at 300 x* 10^6 *since by agarose gel electrophoresis these P-2 plasmids are the same size as pMG1 (molecular weight 312 x* 10^6*) and pMG5 (molecular weight 280 x* 10^6*) (9).*

[b] *Not yet visualized.*

system as a Tra^- plasmid from Dublin (17) and could belong to the same Inc group.

Studies on the mechanisms of gentamicin resistance determined by these plasmids are still preliminary. By substrate profile several different types of AAC and AAD appear to be made. Further study of those plasmids that seem not to make gentamicin modifying activity may also reveal novel mechanisms for aminoglycoside resistance.

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DISCUSSION

Unidentified I: Do you know whether elastase production is plasmid-determined, and is it a factor in virulence?

JACOBY: That is one of the enzymes that *P. aeruginosa* makes. I don't know whether it's an important factor in virulence, although it may be, and I have not specifically looked to see if it is plasmid-determined. I have looked at our set of plasmid-containing strains to see if there has been an effect on protease or nuclease production. The common recipient used made a protease which wasn't turned off by the different plasmids that I had in it. I probably couldn't detect a new protease that might have been made, and I really don't know about the elastase.

Unidentified I: Could it be a result of lysogenization?

JACOBY: Certainly, I don't know whether it's a chromosomal gene. Exotoxin A which seems so important in pathogenicity seems to be [determined by] a chromosomal gene. It has the same mechanism of action as diptheria toxin, and like diptheria toxin its production is regulated by iron, but it seems not to be associated with a phage.

Behme: Is *Bacteroides fragilis* susceptible to the antibiotic HR756; and does the presence of plasmids in *P. aeruginosa* interfere with typing?

JACOBY: I don't know about the susceptibility of *Bacteroides* to the HR756. I think that there are enzymes in *B. fragilis* that are able to deal with it. As for typing, no effects on serotyping have yet been demonstrated. There are effects on both bacteriophage and bacteriocin typing, in that some plasmids give resistance to bacteriophage and bacteriocins, and some also will shut down bacteriocin production.

Lederberg: I'm interested in your high molecular weight plasmids that are non-transferable. I wondered if you ever attempted to shear the molecule into smaller components which could be characterized perhaps differently, and then could be introduced via transformation or conjugation. Also perhaps the markers can segregate if you mechanically made smaller molecules, or if you can mutagenise segregants. This has been done with R26 [IncP1].

JACOBY: Well, everybody who has tried to isolate the 300 M dalton IncP2 plasmids has been able to generate sheared derivatives without any trouble at all. People have tried to transform using IncP2 plasmid DNA and I'm not aware of any success.

Shapiro: Ron Olsen claims to have made small derivatives using restriction enzymes.

JACOBY: That's all I can tell you about it.

Lederberg: There must be a lot of genes not coding for . . .

JACOBY: Sure, there's a lot more DNA there than we can presently account for.

Marrie: I was surprised to see that 19% of your Pseudomonads were resistant to amikacin, and only 6% were resistant to tobramycin. How much amikacin and for how long has it been used to replace other aminoglycosides?

JACOBY: That's an overestimate because if broth MIC's are done it's not that high. Amikacin is restricted in our hospital, but we have observed the emergence of amikacin resistance really without using it that much. This is presumably because the use of other drugs is selecting for cells that aren't permeable to aminoglycosides or that have the few enzymatic mechanisms that can deal with amikacin.

Lederberg: Sometimes in *Pseudomonas* we've found strains that could not be used as conjugation donors at 32°C or 37°C but if you lowered the temperature, markers could be expressed and conjugation would occur. I wonder if that approach has been tried for some of the transfer-less strains?

JACOBY: I haven't done that with the group that I've been looking at for gentamicin resistance transfer. I have looked at altered temperature with a few Tra$^-$ plasmids and not noted it, but I think that's a good suggestion.

Shapiro: Just one other comment along those lines. We found that lysogeny in the donor can often be a bar to transfer.

JACOBY: Yes, I think anything that knocks off either donor or recipient, be it phage or bacteriocin, can interfere with transfer. So some of those could have Tra$^+$ plasmids but be lysogenic for phages which interfere.

Novick: A lot of people have published reports of just resistance surveys and decided whether there was plasmid linkage or not just based on transferability as you mentioned earlier, and I wonder if you would comment on the advisibility of always coupling a transferability assay with at least a screening gel.

JACOBY: I think you've commented on it already.

Novick: The reason I say this is because you could say that what you really want to know is whether the resistance is transferable or not, rather than whether it's on a plasmid.

JACOBY: Well, I think both of them can give you information and you could well have chromosomal genes that were transferred. They haven't turned up very often anyplace, but in principle they could exist. We'd like to find them in *Pseudomonas*, we haven't found any yet. So I think really you need both kinds of information.

INCIDENCE AND PROPERTIES OF R-PLASMIDS OCCURRING IN *SALMONELLAE* IN CANADA

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A recent cross-Canada survey indicated that 36% of the *Salmonellae* from human sources and 33% of those from non-human sources were resistant to one or more antibiotic substances (3). Since the food chain is a major source of human *Salmonellae* infection (6,7) and, because antibiotic resistance genes are frequently associated with conjugative plasmid DNA (5), we began a study of the incidence and types of R-plasmids occurring in *Salmonellae* commonly associated with the human food chain in this country. In addition, we wanted to assess the relatedness of such R-plasmids to determine their common origin(s) or mobility or both within the food chain.

In total, 1787 *Salmonellae* isolated from animal feeds, food animals and humans across Canada were screened for their resistance to thirteen different antibiotics. Eleven serovars were examined: *S. bredeney*, *S. heidelberg*, *S. infantis*, *S. kentucky*, *S. london*, *S. montevideo*, *S. newington*, *S. schwarzengrund*, *S. senftenberg*, *S. thompson* and *S. typhimurium*. All of these were either commonly associated with the entire food chain or overlapped from one source to another. With the exceptions of *S. kentucky* and *S. newington*, R-plasmids were found in all serovars.

The resistance data are summarized in Table 1. The variation in numbers of isolates screened reflects the number of submissions made to the National Enteric Reference Centre from each source during a six month period in 1977-78. Overall, bacteria in animal feeds and feed additives displayed a low incidence of resistance relative to bacteria in food animals and humans (Table 1). The former did, however, show

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TABLE 1. Incidence of Antibiotic Resistant Salmonellae

Source of bacteria	*Number of isolates screened*	*Frequency of drug resistance*[a]	*Resistance (%)* *Multiple*[b]	*Resistance (%)* *(Most common/ largest combination)*
Feeds/ additives	*123*	*11*	*31*	*(2/3)*
Animals	*274*	*34*	*52*	*(2/8)*
Humans	*1390*	*23*	*62*	*(2/9)*

[a] *Percentage of total isolates screened.*
[b] *Percentage of resistant isolates insensitive to two or more antibiotics.*

considerable multiple resistance (31%) with groups of 2 being the most common. Although lower in overall resistance than isolates from animals, bacteria from human sources carried a larger proportion of multiples. The tetracycline-streptomycin couplet was most common in bacteria from animals; whereas ampicillin-streptomycin occurred most frequently in bacteria from humans. The most common resistance found in bacteria from feed was to streptomycin followed by resistance to sulfadiazine; in bacteria from animals resistance to tetracycline was followed by streptomycin; and in bacteria from humans resistance to streptomycin followed by tetracycline.

A representative number of the resistant strains sampled from each source were tested for the possible plasmid location of their resistance determinants. Parameters such as marker stability, phage sensitivity and levels of resistance were monitored, but conjugation studies proved the most fruitful. These data are given in Table 2.

TABLE 2. Frequencies of Drug-Resistant Bacteria with Plasmid-Borne Resistance Determinants: Conjugation Analysis

Source of bacteria	*Number of resistant isolates screened*	*Number carrying conjugative plasmids (%)*	*Number with mobilizable plasmids (%)*	*Total %*
Feed/ additives	*12*	*5(42)*	*4(33)*	*75*
Animals	*43*	*30(70)*	*10(24)*	*94*
Human	*38*	*33(87)*	*2(5)*	*92*

Screening of the human group is not yet complete. The conjugative ability of resistance determinants was measured by crossing each test strain with standard *Salmonellae* and *E. coli* recipients. Five of the twelve strains (42%) from feed sources carried conjugative R-plasmids. The corresponding values for strains from animal and human sources were 70 and 86% respectively. Although limited in their use by the possibility of translocation (e.g., from chromosome to plasmid; 2), mobilization studies enabled us to detect an additional 16 non-conjugative plasmids. Hence, a high proportion of the isolates from each source carried their resistance determinants on conjugative plasmids.

All of the conjugative R-factors detected were classified according to their incompatibility reactions. This was achieved by mating each isolate with a battery of *E. coli* carrying standard plasmids representing the various incompatibility groups, and then the assortment of incoming and resident markers was monitored during growth of the resulting transconjugants in non-selective media. The results are summarized in Table 3. Where possible, the classification was confirmed using plasmid-specific phage or by testing for thermosensitive transfer. Three observations can be made: (1) certain incompatibility types were common to all three sources, i.e., Iα, N, H; (2) while the Iα type was encountered most frequently in strains isolated from feeds and animals, H-types were more common in bacteria from humans; (3) the C-type R-plasmid, found second most frequently in bacteria from animals, was not detected in bacteria from feeds or humans. All the C plasmids were in bacteria from poultry or bovine sources and all determined resistance to streptomycin (and most also to ampicillin).

Table 4 describes some of the R-plasmids detected in this study. The examples given were deliberately chosen to indicate the variety of serovars, antibiograms and plasmid numbers encountered. The trimethoprim resistance determinant in the second last strain displays properties which suggest that it may be located on a transposon.

A similar antibiogram and membership in the same incompatibility group may indicate a common origin for some R-plasmids (1,4). As a further step in establishing their relatedness (or "clonal nature"), we have recently begun to characterize and compare a selected group of R-plasmids according to certain of their physical properties. This is illustrated by the examples given in Table 5. Here the R-factors from four ampicillin-streptomycin resistant *Salmonellae* from different links in the food chain were analyzed according to their incompatibility grouping, phage-sensitivity and molecular weights. Three of the strains carried plasmids belonging to the N

TABLE 3. *Incompatbility Group Distribution of R-Plasmids Detected in* Salmonellae

Source	*Incompatibility Group*	*Number of R-plasmids*
Feeds/additives	*I*α	*3*
	FIV	*1*
	N	*1*
	H_2	*1*
Animals	*I*α	*11*
	C	*6*
	FII	*5*
	H_2	*3*
	*I*γ	*3*
	H_1	*1*
	N	*1*
	FIV	*1*
Humans	H_2	*4*
	FII	*2*
	*I*γ	*1*
	H_1	*1*
	N	*1*
	P	*1*
	*I*α	*1*

TABLE 4. *Antibiotic Resistance Plasmids Occurring in* Salmonellae *in Canada*

Serovar	*Source*	*Antibiogram*[a]	*Plasmids (p)*[b]	*Incompatibility Group*[c]
S. infantis	*Hog supplement*	*SuT*	*pSu, pT**	*I*α*, ?*
S. heidelberg	*Feed manure*	*KNS*	*pKNS*	*FIV*
S. montevideo	*Porcine*	*ST*	*pS, pT**	*FIV, ?*
S. thompson	*Poultry*	*S*	*pS*	*I*α
S. typhimurium	*Bovine*	*ACTSuKNS*	*pA pCTSu, pKNS**	*FII*, H_2*, ?*
S. heidelberg	*Human*	*A*	*pA*	*I*α
S. schwarzengrund	*Human*	*SuTS*	*pSuT, pS**	*P, ?*
S. typhimurium	*Human*	*CSSuKN*	*pCSSuKN*	*FII*
S. typhimurium	*Human*	*ASSuTTp*	*pASSu, pT**	*N, ?*
S. typhimurium	*Human*	*CTSuSNitro*	*pCTSu, pS*, pNitro**	H_1*, ?*

[a] *Su: sulfadiazine, T: tetracycline, K: kanamycin, N: neomycin, S: streptomycin, A: ampicillin, C: chloramphenicol, Tp: trimethoprim, Nitro: nitrofurantoin*

[b] **: depends on other plasmid(s) for conjugal transfer*

[c] *?: unable to determine*

TABLE 5. *Comparison of R-Plasmids from Various Sources*

Host strain	*Serovar*	*Source*	*Province*	*R-Plasmids*			
				Resistances[a]	*Inc. group*	*IKe*[b]	*M.W. (x10⁶)*[c]
221	S. infantis	*Feed manure*	*Manitoba*	*AS*	*N*	*s*	37.5±0.3
1327	S. typhimurium	*Turkey*	*Nova Scotia*	*AS*	*N*	*s*	38.4±0.5
1163	S. typhimurium	*Bovine*	*Ontario*	*AS*	*N*	*r*	9.6±0.4
11523	S. typhimurium	*Human*	*Alberta*	*AS*	*Iα*	*r*	-

[a] *A: ampicillin; S: streptomycin*
[b] *s: sensitive; r: resistant*
[c] *Molecular weights determined by agarose gel electrophoresis*

incompatibility group. The fourth strain, 11523, bore an Iα plasmid and was not further investigated. Only two of the former were sensitive to the N-specific phage, IKe. The third strain, 1163, was IKe resistant. N-type plasmids of this nature were also detected by Anderson (1) in South American isolates. The N-plasmid of strain 1163 also differed from those of strains 221 and 1327 in its molecular size: 9.6 vs 37.5 and 38.4 megadaltons. The similarity in molecular weight of the latter two plasmids is striking.

We interpret the data presented in Table 5 as providing an indication of both the diversity of plasmids and uniformity of the plasmid pool in the food chain. In particular, strains 221 and 1327, of different serovars from different sources and widely separated geographical locations, have been found to carry plasmids that, within the limits of the parameters measured here, appear to be identical. To generate additional support for this conclusion, we are presently preparing restriction endonuclease profiles of these plasmids. This molecular type of analysis is also being extend to several other R-plasmids detected in food chain-associated *Salmonellae*.

It is too early to speculate whether we are dealing with a common reservoir of R-plasmids. However, since feed manure is largely of poultry origin the data presented in Table 5 most likely represent the transfer of plasmid DNA along the food chain. Thus, turkey litter and its associated bacteria is collected in Nova Scotia and then shipped to Manitoba for use as a feed supplement.

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DISCUSSION

Taylor: What temperature did you use to do the initial transfer from *Salmonella*?
BEZANSON: By and large, 37°C. However, if chloramphenicol resistance was involved then they were done at both 26°C and 37°C because chloramphenicol seems to be associated with the H plasmids (which are temperature-sensitive for transfer).
Shapiro: Was the transfer of nitrofurantoin resistance based just on conjugation, because obviously you could have chromosomal gene mobilization occurring?
BEZANSON: It was on conjugation data. If it had been mobilization or translocation from the chromosome into the plasmid, then one would have expected co-transfer of the other drug resistance markers with the nitrofurantoin resistance from transconjugants, but I didn't get that.
Lederberg: You have strains resistant to up to about 5 antibiotics, many of which fall into patterns, and I think maybe you could get more differentiation of the different types if you had a broader spectrum of testing. For instance, streptomycin resistance plasmids may be divided into two categories: some give resistance to spectinomycin and others do not. How many antibiotics did you test?
BEZANSON: We're testing 13.
Lederberg: That's a good spectrum.
BEZANSON: Yes, unfortunately though spectinomycin is not one of those.
Lederberg: Similarly with kanomycin resistance plasmids: some give resistance to neomycin as well.
BEZANSON: Yes, we have done that.
Falkow: There is a difficulty in terms of human strains that have come from a single source outbreak where a significant number of these patients were treated with an antibiotic, and then the strains are submitted for further work and that will obviously change things. I wonder if you have any idea of the number of strains that you would get where you have a single outbreak and these are acquired within the community without medical intervention.

BEZANSON: That's very difficult. Theoretically no *Salmonella* infection except *typhi* should be treated with antibiotics, but that is why I included the outbreak material. I would say a good 75% of them came from clinical situations where, most likely, there had been some antibiotic treatment involved. There was no treatment involved in the single outbreak that had the large resistance pattern. The index case in that incidence was resistant to tetracycline, chloramphenicol and streptomycin.
Falkow: That was an H outbreak?
BEZANSON: Yes.

MULTIPLE ANTIBIOTIC RESISTANCE PLASMIDS IN *Haemophilus influenzae*[1]

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The acquisition of transferable antibiotic resistance appears to have been a recent event in *Haemophilus influenzae* (4,5,12,14,15), and two explanatory hypotheses have been advanced: (i) that strains of *Haemophilus influenzae* have received and maintained R-plasmids from enteric bacteria; (ii) that *H. influenzae* carries resident plasmids that have received translocation elements in abortive matings with other genera or species (5,11). Base sequence relationships between *Haemophilus* R-plasmids carrying different resistance determinants and isolated in different parts of the world suggest either a common incompatibility group or a restricted number of resident plasmids capable of accepting transposable resistance determinants (9,11). Base composition studies of *Haemophilus* R-plasmids support the resident plasmid hypothesis but no putative resident cryptic plasmid has been found to date (9). Recent reports of multiple antibiotic resistance plasmids indicate that, regardless of the mechanism by which R-plasmids were originally established in *H. influenzae*, they are continuing to show genetic rearrangements or acquisition of additional resistance determinants from outside the species, and this is of particular concern to physicians involved in the therapeutic management of patients with serious infections caused by this organism (2,6).

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SOURCE AND CHARACTERIZATION OF STRAINS

In Winnipeg, since 1975, we have been looking at antibiotic resistant strains of the genus *Haemophilus* in relationship to biotype. We have isolated over 100 strains of *H. influenzae* which were resistant to one or more of the following antibiotics: ampicillin, tetracycline, chloramphenicol or kanamycin. Resistance to these antibiotics has previously been shown to be mediated by plasmids in this genus (4,7,14, 15). Based on a limited number of biochemical characteristics (8) these isolates have been classified into biotypes and further characterized as to their ability to transfer resistance determinants and their plasmid composition. The studies reported here primarily cover our results with strains resistant to more than one antibiotic.

Table 1 shows the distribution of antibiotic resistance according to biotype for 100 antibiotic-resistant strains of *H. influenzae*. The distribution of ampicillin-resistant isolates by biotype is no longer significantly different from the distribution seen for sensitive isolates as was originally reported (1). The distribution of tetracycline-resistant isolates, however, remains significantly different from that of sensitive isolates. All multiply resistant isolates carry the tetracycline resistance determinant. In a recent study, Jahn et al. (6) demonstrated the integration of the ampicillin and chloramphenicol translocation segments into the tetracycline transposon in the multiply resistant strains. The single isolate resistant to ampicillin, tetracycline and chloramphenicol reported here was kindly provided by L. Bryan, Calgary (2) and is the only strain not isolated in Winnipeg. Finally, the three strains resistant to both ampicillin and tetracycline were of three different biotypes, indicating either *de novo* occurrences of Ap^r-Tc^r or their acquisition by independent mating events, rather than three separate isolations of the same Ap^r-Tc^r strain.

The age distribution of patients from whom the resistant strains were isolated is shown in Figure 1. Ten of the 14 tetracycline-resistant strains were from patients over 12 years of age. The age distribution of patients with ampicillin-resistant isolates is significantly different from the age distribution of patients with tetracycline-resistant

Table 1. Distribution of Antibiotic Resistance According to Biotype for 100 Antibiotic-Resistant Strains of Haemophilus influenzae

Antibiotic	*Biotype*						
resistance	*I*	*II*	*III*	*IV*	*V*	*Non-I-V*	*Totals*
Ap[a]	31	37	8	3	1	1	81
Tc[b]	0	14	0	0	0	0	14
Ap, Tc	1	0	1	0	1	0	3
Tc, Cm[c]	0	1	0	0	0	0	1
Ap, Tc, Cm[d]	0	0	1	0	0	0	1
Totals	32	52	10	3	2	1	100

[a] *Ap, ampicillin*

[b] *Tc, tetracycline*

[c] *Cm, chloramphenicol*

[d] *Strain UAHf-1, previously reported by L. Bryan (2)*

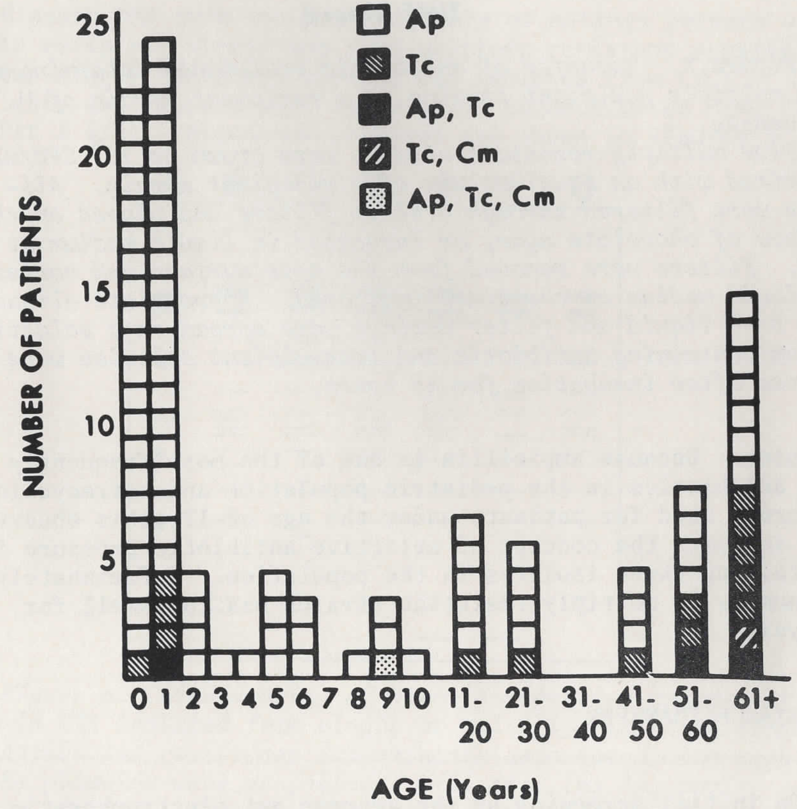

Figure 1. Age distribution of patients from whom resistant strains of H. influenzae *were isolated.*

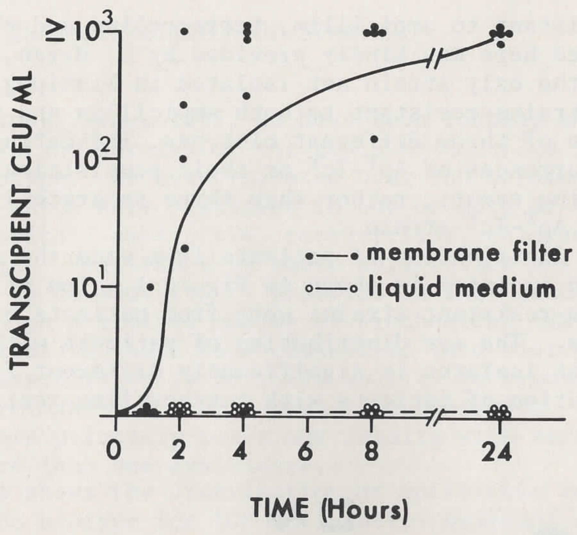

Figure 2. Transfer of antibiotic resistance determinants from multiple resistant strains to a recipient strain of H. influenzae.

Five multiply resistant strains were grown to 10^8 *CFU/ml and mixed with an equal volume of a recipient strain. Aliquots were filtered through 0.45 µm filters and placed on the surface of chocolate agar, or incubated in liquid medium at 37°C. Filters were removed from the agar surface and vortexed in liquid medium at the times indicated. Appropriate aliquots from both liquid and filter matings were spread on a selective medium containing antibiotic and transcipient colonies were counted after incubation for 18 hours.*

isolates. Because ampicillin is one of the most frequently used antibiotics in the pediatric population and tetracycline is rarely used for patients under the age of 12, this observation supports the concept of selective antibiotic pressure in maintaining these isolates in the population. Unfortunately, the number of multiply resistant strains was too small for analysis.

RESISTANCE TRANSFER

On initial screening by the agarose gel electrophoretic method for detection of plasmid DNA described by Meyers et al. (10), 3 of the 5 multiply resistant strains contained a 30-40 Mdal plasmid. Because two of our multiply resistant strains showed no identifiable plasmid on the initial screen, and because we were concerned about strain specific modification, all five multiple resistance determinants were transferred to a single recipient strain, as shown in Figure 2. All five strains co-transferred the non-selected resistance determinant. This supported the hypothesis that they reside on the same plasmid. The failure to transfer resistance determinants in liquid matings suggested a requirement for close cell-to-cell contact, and supported other studies which suggested that conjugation was the mechanism of transfer, although transfer of chromosomal markers by conjugation has not been reported for *H. influenzae.*

CHARACTERIZATION OF MULTIPLE RESISTANCE PLASMIDS

Plasmid DNA from the transcipients of matings between a single recipient strain and five multiply resistant strains and two singly resistant strains was isolated from CsCl gradients and cleaved with restriction endonucleases Hinc II and Pst I (13). Digestion patterns are shown in Figures 3

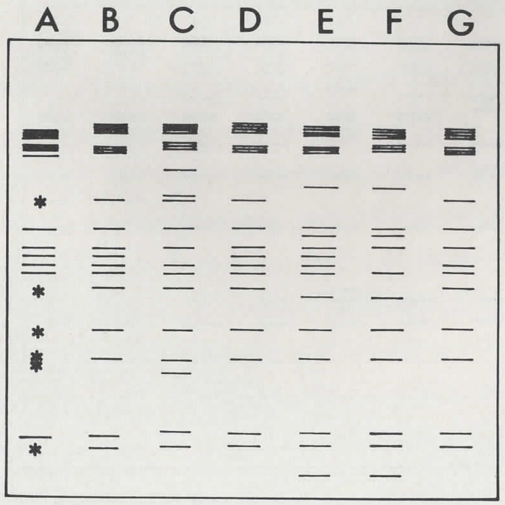

Figure 3. Line drawing of Hinc II digestion pattern of plasmid DNA isolated from singly or multiply resistant strain.

*Antibiotic resistance determinants associated with each of the plasmids were as follows: (A) Ap; (B) Ap, Tc; (C) Ap, Tc; (D) Ap, Tc; (E) Ap, Tc, Cm; (F) Tc, Cm; (G) Tc. Six fragments indicated by an * were present on digestion of plasmid DNA from the donor strain but were lost upon transfer to the recipient strain.*

and 4. Six Hinc II and one Pst I fragments were lost from the ampicillin-resistance donor plasmid on transfer to the recipient strain. Pattern changes have been previously reported by Causey and Brown (3) and emphasize the limitations in using restriction endonucleases for plasmid comparisons.

Overall, however, the digestion patterns suggested significant relatedness between plasmids with multiple resistance determinants and those with single resistance determinants. This is consistent with the previously reported homology found by hybridization studies (6,11).

INCOMPATIBILITY STUDIES

Because of the relatedness between plasmids with multiple resistance determinants and those with single resistance determinants, incompatibility studies were carried out by mating five ampicillin-resistant biotype I strains with five tetracycline-resistant biotype II strains and selecting for ampicillin-tetracycline resistance. Nineteen transcipient strains were isolated and 14 of the 19 transcipient strains were biotype II.

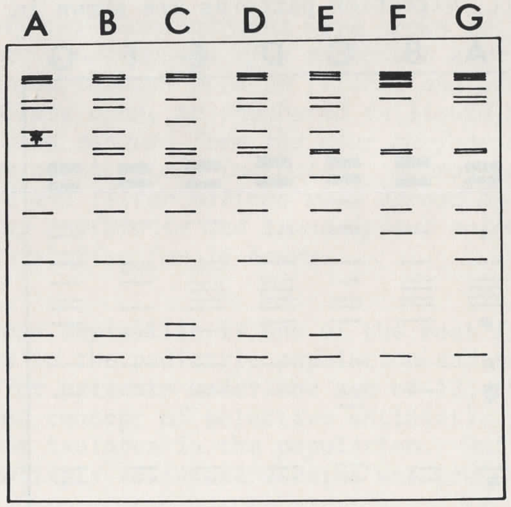

Figure 4. Line drawing of Pst I digestion pattern of plasmid DNA isolated from singly or multiply resistant strains.

*Antibiotic resistance determinants associated with each of the plasmids were as follows: (A) Ap; (B) Ap, Tc; (C) Ap, Tc; (D) Ap, Tc; (E) Ap, Tc, Cm; (F) Tc, Cm; (G) Tc. The single fragment indicated by an * was present on digestion of plasmid DNA from the donor strain but was lost upon transfer to the recipient strain.*

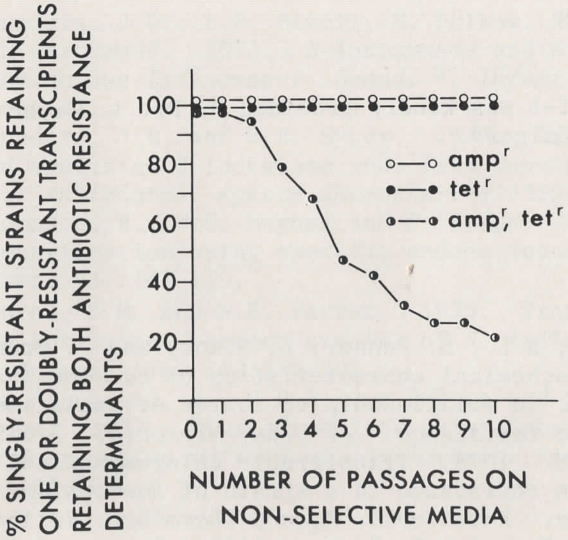

Figure 5. Stability of antibiotic resistance determinants in singly resistant donor strains and multiply resistant transcipient strains.

Five amp^r biotype I strains were mated with five tet^r biotype II strains by co-growth on chocolate agar, and multiply resistant transcipient strains were isolated on selective antibiotic medium. Single colonies were passaged on non-selective chocolate agar and plated on media containing antibiotic at each passage.

The stability of the single and multiple resistance determinants on passage on non-selective media is shown in Figure 5. Multiple antibiotic resistance is rapidly lost on non-selective media, suggesting incompatibility between different plasmids. Strains maintaining multiple resistance determinants after passage on non-selective media demonstrated both linked and non-linked transfer of resistance markers when mated with selected recipient strains. This might indicate the presence of plasmids belonging to at least two different incompatibility groups.

In summary, although multiple antibiotic resistance in *Haemophilus influenzae* is thus far uncommon, biotyping results suggest the continuing evolution of resistance plasmids in this species. Restriction endonuclease digestion patterns and incompatibility studies suggest significant relatedness between resistance plasmids and perhaps a limited number of incompatibility groups. Directional transfer studies and further incompatibility studies may shed additional light on the possible origin and evolution of multiple resistance plasmids in *Haemophilus influenzae*.

ACKNOWLEDGMENTS

Strain UAHf-1 was kindly provided by Dr. L. Bryan of the University of Calgary.

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DISCUSSION

Falkow: Did you say that the *Haemophilus* strains that were multiply resistant were more common in patients that were greater than 12 years of age?

ALBRITTON: Just tetracycline resistance; we don't have enough numbers to do a statistical analysis on the multiply resistant strains.

Falkow: That would be associated with patients with what kind of disease?

ALBRITTON: In the adults, with respiratory disease; usually chronic bronchitis and pneumonia.

Silver: Do you know whether loss or acquisition of a multiple resistance plasmid affects the biotype of the strain?

ALBRITTON: In these particular studies, no. In one experiment where we did 625 matings, we found maybe 5 or 6 of these transconjugants that had neither the biotype of the donor nor the recipient. We lost a selected enzyme function that we were looking at, but in general, the biotype pattern remains constant.

Elwell: Have you been able to relate plasmids in *Haemophilus ducreyi* with those in *H. influenzae?*

ALBRITTON: The 6 Mdal plasmid that we described from *H. ducreyi* has a great deal of homology with the plasmid from *Neisseria*; it also has homology with the 0885 strain of *H. parainfluenzae*, but we haven't done any other specific tests.

Lederberg: Have you got a plasmid DNA transformation system?

ALBRITTON: We've been transforming the small plasmids but not the large conjugative plasmids into *E. coli*.

Lederberg: Do you get the same patterns of incompatibility in *E. coli* that you have with the different types?

ALBRITTON: We haven't looked. We cannot transform the large conjugative plasmids into *E. coli* and we use transformation into *E. coli* only to isolate the non-conjugative plasmids.

Novick: Do you have an explanation for why you could not detect plasmids in some of those strains?

ALBRITTON: We have a whole lot of explanations but . . .

Novick: But you do see supercoiled DNA in the transconjugants?

ALBRITTON: In the transconjugants, yes. We think in the donor they may be as relaxation complexes. At least that's one possibility.

Novick: You would see those in the gels?

ALBRITTON: Well, if you nick our large plasmids to an open circular form, they migrate with the chromosomal DNA and we can't separate them. We're now looking for ways to do that.

Falkow: When you harvest the organisms to do your plasmid preparations, do you grow them on solid media containing antibiotics or do you grow them in liquid?

ALBRITTON: We've done them both ways.

Falkow: And even then you can't find the plasmid?

ALBRITTON: No.

Falkow: We've recently received about 12 cultures from Sweden which sounded very much the way yours do, and by keeping them on solid medium, keeping selective pressure on at all times, we could show supercoiled DNA in 11 of the 12 and they turned out to be run-of-the-mill big plasmids, at least for the ampicillin resistance.

POLYPEPTIDES EXPRESSED BY PLASMID DNA SEQUENCES MEDIATING TRIMETHOPRIM RESISTANCE

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The target site of the antibacterial agent trimethoprim (Tp) is dihydrofolate reductase (DHFR), an enzyme in the folate pathway, which coverts dihydrofolate to tetrahydrofolate (2,9). The mechanism of bacterial resistance to trimethoprim mediated by R plasmids resides in the synthesis of a novel dihydrofolate reductase which is not inhibited by trimethoprim at levels that inhibit the sensitive enzyme specified by a chromosomal gene. DHFRs specified by R plasmids can be separated into two classes based on different levels of sensitivity to Tp and other antifolate compounds (12). The so-called type I enzyme (encoded by plasmid R483) has a 50% inhibitory concentration (I_{50}) for trimethoprim several thousandfold higher than that required to inhibit the *E. coli* chromosomal DHFR. The type II enzyme (encoded by plasmid R67) is, for all practical purposes, completely insensitive to Tp, having I_{50} values several hundredfold higher than those for the type I enzymes (12).

Of interest is the fact that the type I Tp resistance determinant may reside on a transposable DNA segment (transposon Tn). Tn7 has a molecular weight of 8.5 x 10^6 and contains trimethoprim and streptomycin resistance genes originally described for plasmid R483 (1).

Although the plasmid encoded reductases differ from the chromosomal enzymes in their significantly reduced ability to bind Tp, they have the capacity to bind dihydrofolate. In addition, the plasmid encoded reductases share the same molecular weight of about 36,000, whereas the chromosomal DHFR has a molecular weight of about 18,000. Hence the chromosomal and

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plasmid specified reductases appear to be quite distinct from one another on the basis of molecular weight and enzyme characteristics. These observations lead to questions about the evolutionary origin(s) of the trimethoprim-resistant reductases as well as to the relationships of the plasmid specified enzymes to one another and to the chromosomal enzyme.

Our approach to this question has been to isolate DNA sequences containing the structural genes for both type I and type II plasmid encoded reductases and study the genetic expression of these genes in an *E. coli* minicell system. The hybrid plasmids used in this study were constructed using standard molecular cloning techniques employing the cloning vehicles pBR322 and pSC101 (6). The characteristics of the recombinant plasmids constructed for this study are summarized in Table 1. One hybrid plasmid specifying type I DHFR was constructed by inserting a 2.0 x 10^6 dalton BamHI fragment from plasmid R34 (12) into pSC101. A second type I DHFR hybrid plasmid used was ColE1:Tn7 which was constructed by simply transposing Tn7 from R483 into the recipient replicon, ColE1 (4). The hybrid plasmids specifying type II reductase both contained the resistant DHFR gene originating in plasmid R67 (12). In one plasmid, a 6.5 x 10^6 dalton EcoRI fragment was inserted into pSC101 (pFE413) and in the other, a 1.5 x 10^6 dalton portion of the R67 EcoRI fragment was inserted into pBR322 resulting in the hybrid plasmid pFE364.

DNA isolated from chimeric plasmids encoding both Tp-resistant enzyme types was transformed into the minicell

Table 1. Construction of Hybrid Plasmids Specifying R-Plasmid Dihydrofolate Reductases in the Minicell System

Plasmid	*Enzyme type*	*R-plasmid source*	*Cloning vehicle*	*Apparent[b] subunit MW*
ColE1:Tn7	*I*	*R483*	*None[a]*	*18,000*
pFE383	*I*	*R34*	*pSC101*	*18,000*
pFE413	*II*	*R67*	*pSC101*	*9,000*
pFE364	*II*	*R67*	*pBR322*	*9,000*

[a] *Insertion into ColE1 using R483 which carries Tn7*

[b] *MW of polypeptides synthesized by minicells as analyzed by SDS-polyacrylamide gel electrophoresis*

producing strain, *E. coli* DS410 (3). The synthesis of plasmid specific proteins was monitored by uptake of $[^{35}S]$ methionine. Minicell proteins were fractionated by molecular weight on SDS-polyacrylamide gels and visualized by autoradiography (8,14). Figure 1A represents the plasmid specified proteins encoded by the cloning vehicle pBR322 without inserted DNA. The predominant polypeptide bands migrate in SDS-polyacrylamide gels at positions corresponding to molecular weights 32,000, 30,000, and 27,000 respectively. These represent the various forms of β-lactamase (3). Other smaller polypeptides seen in the autoradiograph are, no doubt, involved either in tetracycline resistance or colicin immunity. The polypeptides specified by pFE364 (containing an insertion between the EcoR1 and BamHI sites of pBR322) are shown in Figure 1B. The positions of the polypeptide bands correspond

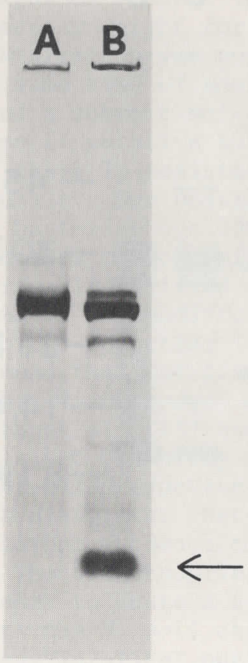

Figure 1. Polypeptides synthesized in minicells harboring pBR322 and pBR322 recombinant plasmids. Polypeptides were labeled with $[^{35}S]$ methionine and analyzed on 15% sodium dodecyl sulfate-polyacrylamide slab gels. Track A shows pBR322 specified polypeptides. Track B shows polypeptides specified by the recombinant pFE364 (type II reductase located on 1.5 x 10^6 dalton R67 DNA fragment inserted in pBR322). Arrow indicates type II reductase subunit.

to those of pBR322 with two additional rather faint bands at 15,000 and 22,000 daltons plus an intense novel polypeptide with an approximate molecular mass of 9,000 daltons. This polypeptide is the putative type II DHFR subunit (13).

In Figure 2, the proteins synthesized in minicells harboring the type I or type II reductase genes are compared. In Figure 2D are the polypeptides synthesized by pFE364 (also shown in Figure 1B). Plasmid pFE413 is pSC101 plus a 6.5 x 10^6 dalton EcoRI fragment insert containing both the reductase gene and the β-lactamase gene from plasmid R67. The polypeptides synthesized by minicells harboring this hybrid plasmid is one of approximately 9,000 daltons in molecular mass presumably identical to that synthesized by pFE364 (Figure 2D).

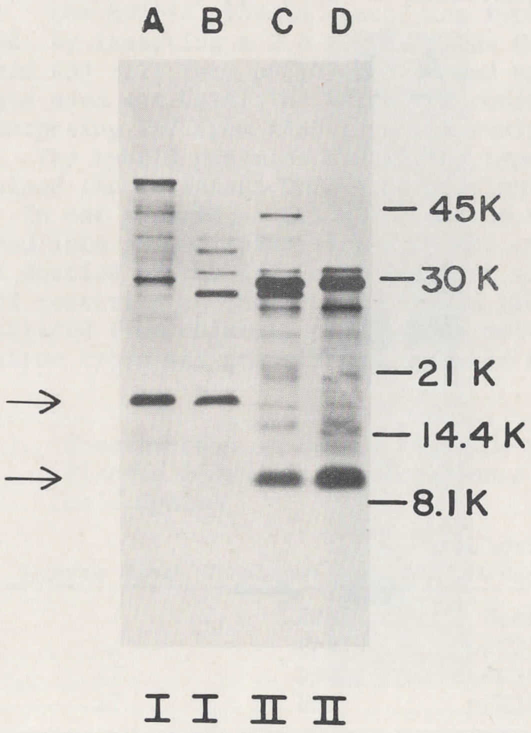

Figure 2. Polypeptides synthesized in minicells harboring recombinant plasmids specifying type I or type II reductase and electrophoresed on a 15% SDS-polyacrylamide slab gel. The hybrid plasmids were ColE1::Tn7(A), pFE383(B), pFE413(C), and pFE364(D). Arrows indicate positions of reductase subunits.

In contrast, the novel polypeptide specified by pFE383 which contains a type I reductase gene (from plasmid R34) has a molecular weight of approximately 18,000 (Figure 2B). The other polypeptide bands seen in Figure 2B are characteristic of the pSC101 cloning vehicle (data not shown). Figure 2A shows the proteins synthesized in minicells harboring the plasmid ColE1:Tn7. Compared with the ColE1 background (data not shown), the major novel polypeptide species specified by this constructed plasmid has a molecular mass of 18,000 daltons and appears to be similar to the novel polypeptide encoded by pFE383.

The results of these experiments suggest that the putative subunits of the type I and type II, plasmid-specified reductases have different molecular weights (Table 1). On the basis of our minicell analysis, the type II subunit has an average molecular weight of 9,000. This is consistent with a reported subunit molecular weight for this enzyme of 8,500 derived from more classical enzyme purification techniques (13). Employing the same kind of analysis for the type I Tp-resistant DHFR we find a subunit molecular weight of 18,000, twice that of the type II putative subunit.

The origin(s) of these Tp-resistant enzymes specified by plasmids remains a puzzle. One possible source is the resistant DHFR encoded by bacteriophage T4. While this phage specified enzyme resembles the plasmid enzyme in some superficial respects, partially purified R483 reductase did not form a precipitin band in immunodiffusion tests against antiserum to T4 dihydrofolate reductase (10). In addition, Mosher et al. (10) found that the plasmid DHFR was not enzymatically inactivated by T4 antibody. An alternative origin was suggested by Smith et al. (13) namely, that the plasmid specified reductase is not primarily a reductase at all, but is rather some type of oxidoreductase that has acquired the ability to reduce dihydrofolate. Recently we have obtained some DNA-DNA hybridization evidence that may lend some support to this hypothesis. The trimethoprim/streptomycin resistance transposon, Tn7, appears to share a significant amount of DNA sequence homology with the *E. coli* chromosome (5). The exact nature of the presumed "Tn7-like" nucleotide sequences in the *E. coli* chromosome is unknown. They could represent known insertion sequences (11) or γδ sequences (7), or the Tn7 transposon might harbor a gene(s) that is universally found in the *E. coli* genome. To speculate further, perhaps the type I plasmid encoded DHFR is structurally related to its chromosomal counterpart. The molecular mass of the Tp-sensitive, chromosomal enzyme is 18,000 (12). The type I DHFR has a molecular weight of 35,000 in non-denaturing gels and an apparent subunit molecular weight of 18,000 in SDS-polyacrylamide gels. It is conceivable that the type I reductase of

R483 (plasmid R483 contains Tn7) is a dimeric form of the chromosomal enzyme that has retained its normal substrate affinity but somehow has been rendered Tp-resistant. This hypothesis can be tested when this plasmid reductase is purified, sequenced and compared with the chromosomally specified DHFR.

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DISCUSSION

Shapiro: What sort of enzyme is coded by Tn402?

FLING: So far it has not been assayed by us. I don't know whether there is information about that, but it doesn't appear to have a gross homology with the Tn7 enzyme.

Novick: It seems to me that a reasonable source for these things might be naturally occurring soil bacteria that have resistance to trimethoprim. I gather that most naturally occurring bacteria are sensitive to trimethoprim, and I wonder if you might screen for them to find a candidate for your enzyme?

FLING: No, we haven't looked at any trimethoprim resistant strains outside of enterobacteriaceae, but maybe we should.

Foster: Is there any similarity in amino acid sequence between types I and II?

FLING: Type I hasn't been sequenced.

CHROMOSOMAL LOCATION OF PLASMID-TYPE RESISTANCE GENES IN *Streptococcus pneumoniae*[1]

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Multiply antibiotic-resistant clinical isolates of *Streptococcus pneumoniae* (pneumococcus) have appeared in South Africa (1), Japan (4), Paris (2), and elsewhere. Some of their properties, including the presence of chloramphenicol acetyltransferase (2,4,5) and the low frequency of transfer of their resistance determinants by transformation (2), suggested that plasmid resistance factors may be present in them, even though no plasmid DNA can be detected (2,3,5,7). We examined the chloramphenicol and tetracycline resistant strains BM6001 from Paris (2) and N77 from Japan (4) and found that the *cat* and *tet* genes were contained in adjacent non-homologous insertions into the chromosomes of both strains.

The evidence for this conclusion was independent of the presence or absence of plasmid DNA in these strains and was based on transformation analysis of the physical properties of the *cat* and *tet* determinants, as follows: (i) *tet* was physically linked to *cat*, as shown by high co-transfer from lysates of *cat tet* donor strains and not from mixed lysates of *cat* and *tet* donors; (ii) *cat* was linked to the chromosomal *nov* gene, as shown by significant co-transfer from unsheared lysates that was prevented by gentle shearing of the lysates; (iii) *cat* donor activity resided with chromosomal markers in dye-buoyancy equilibrium gradients; (iv) *cat* donor activity

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remained together with chromosomal marker activity over a wide range of sedimentation velocities and, most significantly, shifted its velocity distribution with the chromosomal markers as the donor preparation was sheared. These properties held both for *cat tet* strains and for singly transformed *cat* strains. In contrast, donor activity of markers on plasmid *p*IP501 (20 Mdal), which we introduced into pneumococcus from *Streptococcus faecalis* and was readily detected by gel electrophoresis, showed very different behavior in velocity and dye-buoyancy gradients.

The donor activity of *tet* was low and very sensitive to shearing of the donor DNA, implying that it was part of a long insertion, probably $\geq$ 30 kilobases, all of which was necessary for integration to succeed. In contrast, *cat* activity was relatively stable, surviving extensive purification of the DNA and moderate shear forces, and appeared to be associated with a shorter insertion that behaved physically according to the length of the chromosomal fragment in which it was found. This result and the linkage of *cat* to *nov* were incompatible with the hypothesis that *cat* was carried on a plasmid that for unknown reasons could not be detected in the same lysates in which its transforming activity was stable.

The co-transfer of *cat* and *tet* showed a striking asymmetry, in that a majority of *tet* transformants also acquired *cat* but only a few *cat* transformants also acquired *tet*. Another observation was that the presence of one of these determinants in the recipient promoted the incorporation of the second. This demonstrated the enhanced accumulation of heterologous genes by a process that did not involve plasmids in the species of concern. We do not know whether one or both of the *cat* and *tet* insertions were transposons or whether transposition contributed significantly to their transformation. However, most of the behavior of these genes could be accounted for by analogy to transformation of deletion recipients, using legitimate pairing in regions of flanking homology.

These results are described in more detail in Shoemaker et al. (6). To summarize the status of plasmids in pneumococcus, (i) a 2.0 Mdal cryptic plasmid occurs in some laboratory strains (3,7); (ii) larger plasmids from other streptococci can be introduced and maintained in this species; but (iii) no plasmid is present in any drug-resistant clinical strain yet examined.

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DISCUSSION

Shapiro: Have you looked at transformation of *rec*⁻ strains?

SMITH: We don't have any recognisable *rec*⁻ mutants in pneumococcus.

Clewell: Have you tried transforming this DNA into something like *Streptococcus sanguis* which might support it as a plasmid and see if it would circularize?

SMITH: No, we haven't done that.

Falkow: If you're able to put a conjugative plasmid into pneumococcus, might you not then pick up a chloramphenicol resistance gene(s) and then be able to transfer it out?

SMITH: Yes you could, definitely.

Falkow: But you've not done that?

SMITH: No, the exact plasmid I have already has chloramphenicol resistance gene(s) on it, but we're thinking about doing something like that.

Falkow: You said these were similar to the strains that have come out of South Africa in their resistance patterns?

SMITH: I'm guessing at that. All I know is that they look very much the same to me: the M.I.C.'s are the same; they're linked; and they have come from Japan, Paris, and from South Africa. It looks suspicious to me.

PLASMID MEDIATED CLINDAMYCIN RESISTANCE IN *Bacteroides fragilis*[1]

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The obligately anaerobic bacteria of the genus *Bacteroides* are normal inhabitants of the gut and oral cavities of man and other animals. This genus is divided into 5 species: *fragilis*, *thetaiotaomicron*, *vulgatus*, *distasonis* and *ovatus*. Many strains are present in the range of 1 x 10^{10} to 5 x 10^{10} organisms per gram of feces, and out-number the facultative anaerobic bacteria (also present in the human intestinal tract) by 1000 to 10,000 fold. The significance of *Bacteroides* as an opportunistic pathogen is well established and treatment of *Bacteroides* infection is often difficult since many strains are resistant to *penicillin G*, *ampicillin*, *cephalosporins*, and the *aminoglycoside* antibiotics. For several years clindamycin (Cc), a chlorine substituted derivative of lincomycin (Ln), and chloramphenicol (Cm) have been the drugs of choice for anaerobic infection and septicemia.

Recently, clindamycin resistance was demonstrated in clinical isolates of *B. fragilis* (16) and *Clostridium perfringens* (2). It is possible that the widespread use of clindamycin and other antibiotics for treatment of anaerobic infections has resulted in the partial loss of susceptibility to this important antimicrobial agent. Furthermore, some isolates of *B. fragilis* are resistant to erythromycin (Em) and tetracycline (Tc) and the frequency of isolates resistant to Tc is increasing (10,18,8). Mancini and Behme (7) demonstrated the

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apparent transfer of tetracycline resistance from *B. fragilis* to *E. coli* K12 and assumed that a plasmid was responsible for tetracycline resistance. However, this work had serious shortcomings: the individual markers were unstable in the *E. coli* transconjugants and the authors failed to show the existence of covalently closed circular DNA in either the *B. fragilis* or the *E. coli* transconjugants.

There is a more recent report of a conjugative drug resistance (R) plasmid in *B. ochraceus* which is a resident of the oral cavity (5). This R plasmid specified resistance to chloramphenicol, tetracycline and kanamycin. Since these anaerobes are resistant to aminoglycoside antibiotics, it is rather surprising that resistance to kanamycin in *Bacteroides* is mediated by an R plasmid. Welch et al. (21) reported a clinical isolate of *B. fragilis* which could transfer Cc^r, Ln^r, Em^r to other *Bacteroides* strains, and they concluded that these resistances were borne on a conjugative plasmid.

VARIABILITY IN THE PATTERNS OF SUSCEPTIBILITY TO ANTIBIOTICS

In recent months we collected clinical isolates of anaerobic bacteria from several nearby hospitals (Table 1), and minimum inhibitory concentrations (MIC) of antibiotics were determined for each isolate by a modification of the broth method of Wilkins et al. (22). The results of the susceptibility patterns of *B. fragilis* are shown in Table 1. Of the 92 isolates from the area hospitals, 11 were found to have an MIC of >128 μg of clindamycin.

The resistance of 20% of the *B. fragilis* isolates found in two of the Detroit area hospitals may be related to the extremely high use of this antimicrobial agent. Detroit General Hospital (DGH), because of its role in handling trauma and emergency surgery, uses more clindamycin than the other larger hospitals in the Medical Center complex. Harper Hospital, the other hospital that has 20% of its *B. fragilis* isolates resistant to clindamycin, is an oncology hospital where considerable surgery is done. With the development of up to 20% resistance to clindamycin by MIC susceptibility patterns, it is important that this resistance mechanism be further investigated.

Table 1. Comparative Susceptibility Patterns of B. fragilis

Antibiotic		*Source and number of cultures tested*[a]			
Name	*Concn. (μg/ml)*	*HFH 17*	*DGH 31*	*GH 16*	*HH 28*
Clindamycin	8	-	81[b]	80	92
	4	-	78	80	92
	2	-	78	80	92
	1	100	78	80	86
Chloramphenicol	16	-	94	100	96
	8	100	77	82	89
	4	77	58	18	36
	2	71	26	18	0
Doxycycline	8	100	94	91	100
	4	88	81	82	86
	2	53	65	64	68
	1	47	45	55	50
Tetracycline	8	65	61	55	57
	4	47	35	36	50
	2	47	29	27	39
	1	47	26	27	39
Erythromycin	4	59	71	73	79
	2	47	61	73	58
	1	47	32	55	28
	0.05	24	26	45	21

[a] *HFH, Henry Ford Hospital; DGH, Detroit General Hospital; HGH, Harper-Grace Hospital; HH, Hutzel Hospital*

[b] *Percent susceptible*

IDENTIFICATION OF PLASMID DNA

Six of the isolates reported in Table 1 develop a high level of resistance as seen in *Staphylococci* and *Enterobacteriaceae* (15). In this type of resistance the acquisition of a plasmid (or R factor) results in the production of specific enzymes which degrade or modify the antibiotic (1,6) so that it loses its specificity for its target site in the bacterial cell (9). Several strains of *Bacteroides* were reported to contain extrachromosomal DNA, but the biological function of these plasmids is unknown (3,17,20). We therefore selected 4 strains, three from DGH and one from Henry Ford Hospital (HFH) to test for the presence of plasmid DNA. DNA was extracted from cultures of the clindamycin resistant or sensitive strains in mid-log phase by a modification of our usual procedure (12). Each culture yielded a "satellite" DNA component of higher density than the bulk (chromosomal and open circular) DNA, and this plasmid DNA was examined with the electron microscope (see reference 11). The sizes of plasmid DNA molecules in preparations from the various *B. fragilis* strains resistant to high concentration of clindamycin are listed in Table 2. Two sizes of molecules were predominant in strain DGH10 with contour lengths of 3.5 kb and 5.8 kb. In DGH13, there were 4 size classes. Another clinical isolate, resistant to tetracycline, erythromycin and clindamycin (DGH8) contained at least 4 classes of plasmid DNA: 3.5 kb, 5.8 kb, 18 kb, and a large one. A fourth strain, HFH6 (clindamycin sensitive), contained no plasmid DNA.

In interpreting these findings we shall first consider the difference between the strains. A plasmid molecule with a length of 3.5 kb was present in all clindamycin resistant strains, but absent in clindamycin sensitive strains, either natural isolates or cured (Table 2). The large plasmid present in strain DGH8 seemed to be conjugative and could promote the transfer of drug resistance to a suitable strain of *E. coli* (a *recA* strain would be preferable, to eliminate the possibility of recombinants being produced by chromosomal transfer). We intend to test all Cc^r transcipients for the

Table 2. Bacterial Strains and Relevant Characters

Designation	*Phenotypes*[a]	*Plasmid size (kb)*
DGH10	$Cc^rLn^rEm^rTc^s$	*3.5 kb (25)*[b]*, 5.8 kb (22)*
DGH13	$Cc^rLn^rEm^rTc^s$	*3.5 kb (22), 5.8 kb (20),* *12.0 kb (17), 18.0 kb (10),* *34.0 kb (6)*
DGH8	$Cc^rLn^rEm^rTc^r$	*3.6 kb (20), 5.8 kb (24),* *18.0 kb (9), 37.0 kb (5)*
HFH6	$Cc^sLn^sEm^sTc^s$	*no plasmid*
DGH10 (Cured)	$Cc^sLn^sEm^sTc^s$	*no plasmid*

[a] *Cc, clindamycin; Ln, lincomycin; Em, erythromycin; Tc, tetracycline; r, resistance; s, sensitivity*

[b] *Numbers in parentheses represent the total number of molecules measured.*

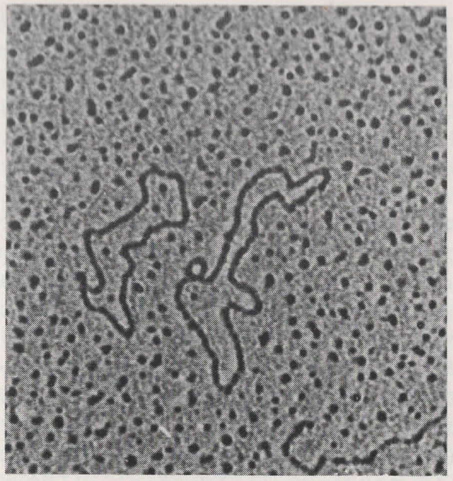

Figure 1A. Electron micrograph of plasmid molecules (3.5 kb and 5.8 kb) isolated from a clindamycin resistant strain, DGH10.

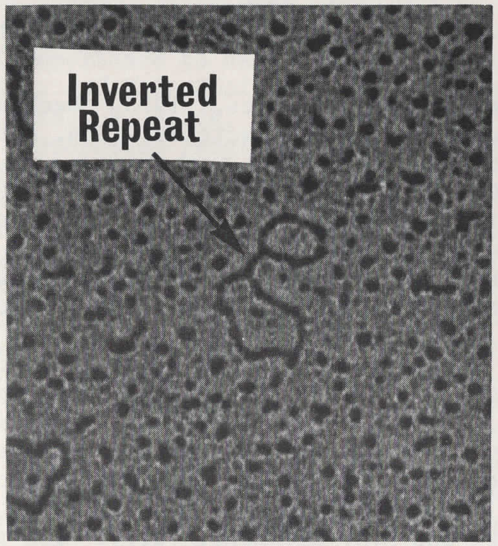

Figure 1B. Electron micrograph of a self-hybridized molecule showing the inverted repeat.

presence of plasmids associated with the resistance genes. Moreover, we are now testing compatibility relationships in one chosen host (4,14).

To explain the presence of multiple circular forms of DNA in all different clindamycin resistant strains, we may consider two possibilities: either all these different sizes represent unrelated functions or these are all plasmids generated from the smallest plasmid (3.5 kb) by some unknown mechanism. The latter possibility appears to be more likely for two separate reasons: (a) the strain, DGH10 showed a number of circular DNA molecules when the growth medium contained clindamycin (256 µg/ml), otherwise only the two sizes 3.5 and 5.8 kb, were seen (Figure 1A); (b) in a heteroduplex experiment, we found complete homology between 3.5 kb molecule and 5.8 kb molecules in addition to a small inverted duplication (Figure 1B) present in self-hybridized molecules (14). Our data combined with those of Tally et al. (19) and Welch et al. (21), may confirm that clindamycin resistance plasmids exist in both conjugative and non-conjugative forms.

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DELETION ANALYSIS OF A TRANSFERABLE *BACTEROIDES* R PLASMID[1]

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The clinical importance of self-transmissible extrachromosomal genetic elements that confer resistance to antibiotics (conjugative R plasmids) in facultatively anaerobic Gram-negative and Gram-positive bacteria is well recognized (2). Only recently has information become available on the existence and properties of R plasmids in obligately anaerobic bacteria. Brefort et al. (1) first reported the presence of conjugative R plasmids in the Gram-positive anaerobe, *Clostridium perfringens*. Guiney and Davis (3) then described an R plasmid from *Bacteroides ochraceus* that was able to transfer kanamycin, tetracycline and chloramphenicol resistance to *E. coli*. In 1979, reports of conjugative antibiotic resistance in *Bacteroides fragilis* appeared from the laboratories of Sebald (8), Talley (9) and Macrina (11). Conjugative resistance to clindamycin, the drug of choice against *Bacteroides* infection, was reported from all three laboratories. In this communication, we describe the general properties of a conjugative plasmid of *Bacteroides fragilis* which is able to confer resistance to lincosamide-macrolide (LM) antibiotics. Further, we describe the preliminary characterization of a derivative of this plasmice which carries a deletion that appears to span some or all of the gene(s) determining resistance to LM antibiotics.

The strains of *B. fragilis* or *B. uniformis* used in this

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work were grown anaerobically according to previously published procedures (11). Filter sterilized antibiotics were used in selective media at the following concentration: clindamycin: 10 µg/ml; rifampicin: 10 µg/ml; and, ampicillin: 1 mg/ml. Conjugative matings and progeny selection were performed as previously described for *Bacteroides* (11). Concentrated, cleared cell lysates were prepared according to the method of Meyers et al. (7) with minor modification (Welch and Macrina, in preparation). Purified plasmid DNA was isolated by dye buoyant density centrifugation (11). Cell lysates or purified plasmid DNA were analyzed electrophoretically using 0.7% agarose slab gels and a tris-borate buffer system (7). Restriction endonuclease enzymes were purchased from Bethesda Research Laboratories ([BRL] Rockville, Maryland). Reaction buffers and conditions for endonuclease digestion were as recommended by BRL.

Our initial findings on plasmid-associated transferable LM-resistance in *Bacteroides* are summarized in Table 1 and Figure 1. *B. fragilis* V479-1 contains a unicopy 27 x 10^6 dalton (27 Mdal) plasmid, pBF4, which is found in progeny cells of matings between strain V479-1 and *B. uniformis* V528. This latter strain is normally plasmid-free and contains a chromosomal mutation conferring rifampicin resistance for counter selection. Lanes B, C and D of Figure 1 respectively illustrate the plasmid content of the V479-1 donor strain, the V528 recipient strain and a typical progeny (designated V544) of a V479-1 x V528 mating selected by plating on clindamycin-rifampicin containing medium. Lane E contains a cleared, concentrated cell lysate of *B. fragilis* V531. This strain is LM

Table 1. Summary of Properties of pBF4

Molecular size (Mdal)[a]	*Copies/ chromosome equivalent*[b]	*Phenotype*[c]	*Host range*	*Origin*
27.2	~1	*Lincosamide-macrolide resistance (>500µg/ml); conjugational-like transmissibility*	Bacteroides fragilis Bacteroides uniformis Bacteroides ovatus Bacteroides thetaiotaomicron Bacteroides fragilis sub-sp. a	*Single colony isolate (designated V479-1) of* B. fragilis *92 resistant to lincosamide-macrolide antibiotics; from M. Sebald (8).*

[a] *As determined by molecular contour length measurements (11)*

[b] *Determined by analysis of* ^{3}*H-thymidine labeled whole cell lysates as previously published (10).*

[c] *Minimum inhibitory concentration of clindamycin determined by colony forming unit assay (5).*

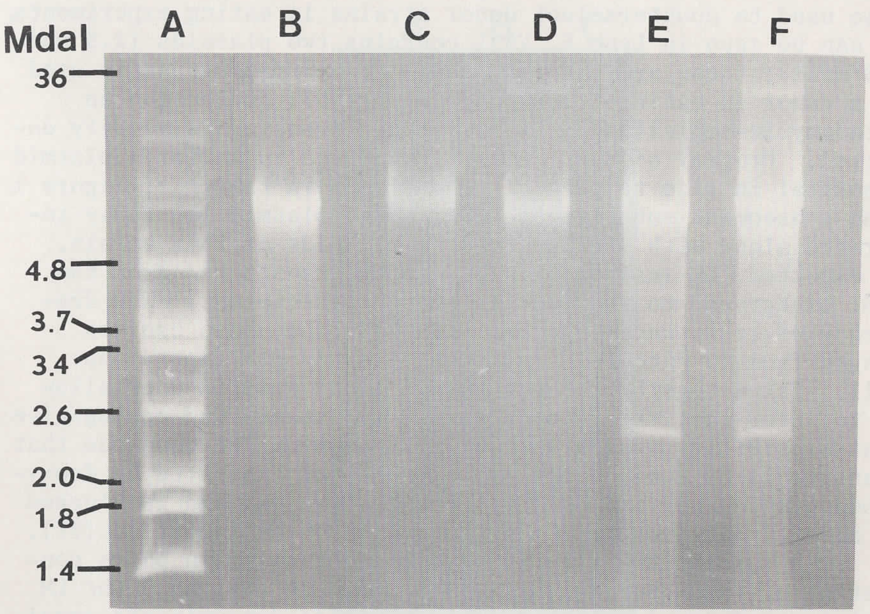

Figure 1. Electrophoretic analysis of Bacteroides *cell lysates. Lane A: Size reference covalently closed circular plasmid molecules from* E. coli *V517 (6); sizes are given to the left of the photograph. Lane B: Cleared, concentrated cell lysate of* B. fragilis *V479-1; the uppermost component is the 27 Mdal pBF4 plasmid while the intensely staining lower component (in the 10-15 Mdal range) represents contaminating chromosomal fragments. Lane C: Lysate of plasmidless* B. uniformis *V528 showing only a chromosomal component. Lane D: Lysate of* B. uniformis *V544 recovered from V479-1 x V528 mating with selection on clindamycin-rifampicin, showing 27 Mdal plasmid. Lane E:* B. fragilis *V531 showing cryptic plasmids of 2.5 and 6.8 Mdal. Lane F: Clindamycin-resistant progeny of* B. uniformis *V544 x* B. fragilis *V531 mating showing the 27 Mdal plasmid along with the two cryptic plasmids. Agarose concentration was 0.7% and electrophoresis is from top to bottom.*

sensitive but resistant to high levels of ampicillin, a trait also used to counterselect donor strains in mating experiments. As can be seen in Lane E, V531 contains two plasmids (2.5 and 6.8 Mdal); these are cryptic. When *B. uniformis* V544 is used as a donor in matings with *B. fragilis* V531 (selection on clindamycin-ampicillin), LM resistance transfer is readily detected. Progeny of such crosses are found to harbor a plasmid identical in size to pBF4 as can be seen in Lane F of Figure 1. Upon subsequent subculture, the 27 Mdal plasmid is stably inherited along with the two cryptic plasmids of this strain. As expected, LM resistance also can be transferred from the V531 background to yet another suitable recipient. The frequency of resistance transfer in all of the above crosses ranged from 10^{-5} to 10^{-6} resistant progeny per input donor cell. Taken together, these physical and genetic data allow us to infer that the gene(s) for LM-resistance and conjugative transmissibility reside on the pBF4 plasmid. We conclude that transfer is by conjugation since it is not inhibited by deoxyribonuclease, nor mediated by donor filtrates, and is blocked by agents that prevent donor and recipient cell contact (11).

Drug susceptibility studies of isogenic strain pairs consisting of V528-V544 and V531-V545 indicate that only the LM resistance determinant is carried by pBF4; determinants specifying resistance to any other classes of antibiotics have not been found to be associated with the pBF4 plasmid.

Attempts to selectively eliminate or cure the pBF4 plasmid and the associated LM resistance phenotype were successful. Following overnight growth of V479-1 in 5 μM ethidium bromide, LM-sensitive variants were detected at a frequency of ~1%. Most of these sensitive clones contained no detectable pBF4 plasmid DNA. During the screening, however, we did detect a LM-sensitive clone which carried a plasmid some 5 Mdal smaller than pBF4. CsCl-ethidium bromide gradient purified preparations of pBF4 and its deletion bearing derivative, designated pBF4Δ1, were examined by 0.7% agarose electrophoretic gel analysis (Figure 2). The migration of the pBF4Δ1 plasmid (Lane C) relative to the marker DNAs (Lane A) and pBF4 (Lane B) indicated a molecular size of approximately 20 Mdal. Contour length determinations of pBF4Δ1 revealed a molecular size of 21.88 ± 0.9 Mdal.

In order to characterize the deletion carried by pBF4Δ1, we subjected it and pBF4 to site specific (restriction) endonuclease digestion. pBF4 digested with EcoR1 (Lane B, Figure 3) yielded 6 fragments (designated A-F) ranging in size from 11.6 to 1.8 Mdal based on migration relative to the *Hind* III fragments of phage lambda DNA. EcoR1 digestion of pBF4Δ1 (Lane C) revealed fragments corresponding to D and F in pBF4 were missing. In addition, the largest EcoR1-generated fragment of pBF4Δ1 was some 0.5 Mdal smaller than fragment A of pBF4.

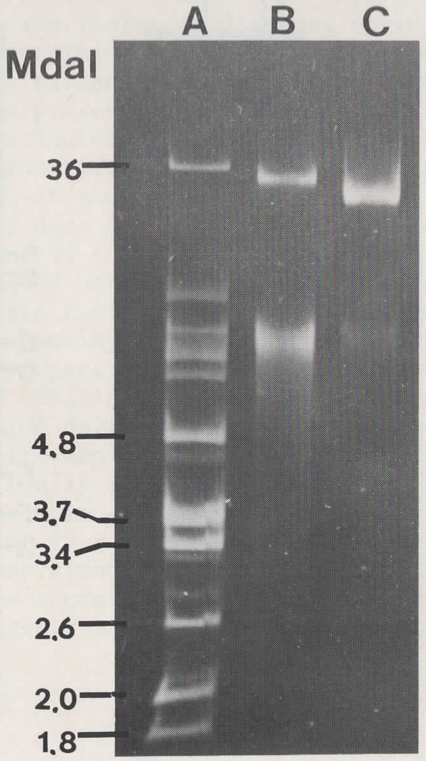

Figure 2. Agarose gel electrophoretic analysis of Bacteroides *plasmids. Covalently closed circular DNA was isolated by dye-buoyant density centrifugation and analyzed directly on a 0.7% agarose gel. Lane A: Size reference plasmid molecules from* E. coli *V517 (6); sizes are given to the left of the photograph. Lane B: pBF4 covalently closed circular DNA, migrating in the 27-30 Mdal range. Lane C: pBF4Δ1 covalently closed circular DNA migrating in the 20-22 Mdal range.*

Thus, the deletion carried by pBF4Δ1 involved the pBF4 EcoR1 fragments corresponding to D, F and a small portion of A. The relationship of pBF4 to pBF4Δ1 was probed further with restriction endonuclease *Hind* III (Lanes D and E, Figure 3). *Hind* III digestion of pBF4 yielded 7 fragments ranging in size from 7.7 to 1.2 Mdal (Lane D). The *Hind* III fragment D of pBF4 was a doublet as revealed by densitometric tracing of a photographic negative of the gel. The pBF4Δ1 *Hind* III digest showed that the deletion carried by this plasmid covered

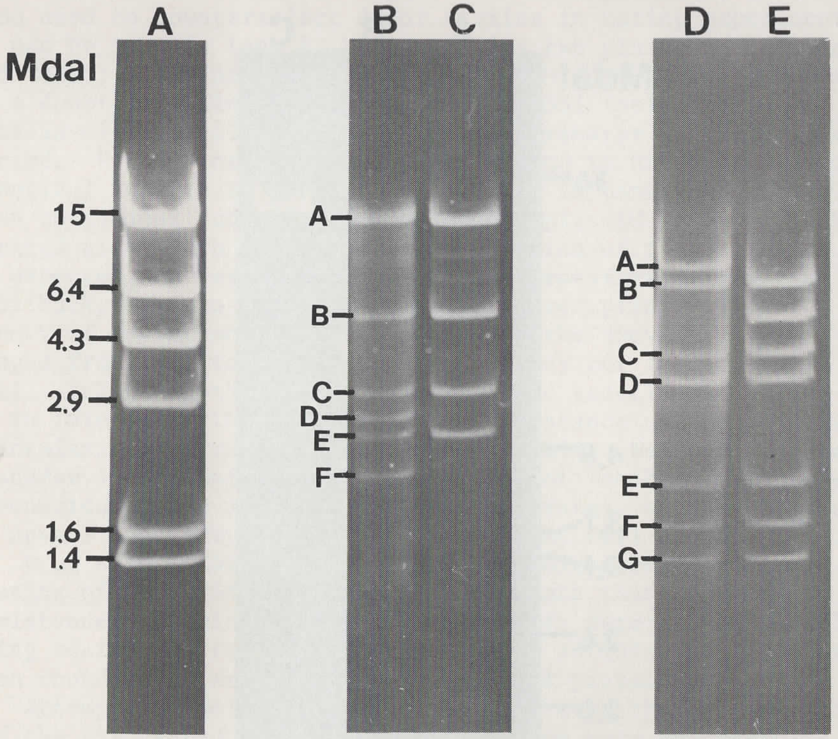

Figure 3. Restriction enzyme analysis of pBF4 and pBF4Δ1. Lane A: Phage lambda DNA digested with Hind *III; sizes of lambda fragments are given to the left of the photograph. Lane B: EcoR1 digest products of pBF4. Lane C: EcoR1 digest products of pBF4Δ1. Lane D:* Hind *III digest products of pBF4. Lane E:* Hind *III digest products of pBF4Δ1.*

approximately 25% of the *Hind* III A fragment as well as one of the fragment members present in the doublet component designated D in the pBF4 digest (Lane D).

Phenotypically, the pBF4Δ1 carrying strain did not revert to LM-resistance spontaneously or following treatment with a variety of mutagens. This indicated the irreversible loss of the resistance determinant(s) and was consistent with the hypothesis that the deletion carried by pBF4Δ1 covered at least a portion of the LM-resistance gene(s). Unfortunately, the absence of any directly selectable markers in the pBF4Δ1-carrying strain precluded a determination of continued conjugational donor proficiency in this strain.

In summary, pBF4 is a self-transmissible plasmid conferring LM-resistance in *Bacteroides*. A derivative of pBF4, designated pBF4Δ1, was isolated following ethidium bromide treatment of a pBF4-containing strain. This strain was recovered as a LM-sensitive variant which did not revert to LM-resistance even in the presence of mutagens. pBF4Δ1 carries a deletion of approximately 5 Mdal and the location of this lesion has been identified by restriction endonuclease analysis (Figure 3). It should be noted that the use of the restriction enzymes does not enable us to rule out independent multiple deletions in the generation of pBF4Δ1. However, preliminary analyses of heteroduplex structures of pBF4 and pBF4Δ1 DNA indicate that the lesion carried by pBF4Δ1 is a single deletion spanning some 23% of the pBF4 molecule (Welch and Macrina, in preparation). We are continuing to isolate and characterize additional deletion mutants of the pBF4 plasmid. Such deletion derivates will provide the means to explore the molecular organization of R plasmids in the clinically and biologically important *Bacteroides* group. pBF4Δ1 and additional deletion-bearing derivatives of pBF4 currently are being used together with a *Bacteroides* R plasmid conferring LM and tetracycline resistance in order to assess whether phenomena such as surface exclusion, plasmid incompatibility and resistance gene translocation are operative in the *Bacteroides* genus.

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DISCUSSION

Clewell: What were the conditions of mating and transfer?

MACRINA: The cells were collected on Millipore filters and incubated for various lengths of time. We know that the transfer appears to occur almost as quickly as we can make the conditions anaerobic. If you collect cells on filters and put them in a gas-pack jar, within 2 hours you can detect transfer and that's roughly about the time it takes to achieve anaerobic conditions in the jar.

Clewell: What sort of frequencies do you get, say after 2 hours?

MACRINA: Roughly around 10^{-7}. However, the matings I showed you were non-isogenic. In isogenic mating pairs which we have actually published data on in the last issue of *Plasmid*, frequencies start off at around 10^{-6} and increase up to 10^{-3}.

Clewell: How long does it take to get, say 10^{-3}?

MACRINA: 6 hours.

Falkow: Does it go in broth?

MACRINA: We've never been able to get it to go in broth.

PLASMIDS ASSOCIATED WITH AN ENTEROTOXIGENIC STRAIN OF *E. coli*

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Enterotoxigenic strains of *Escherichia coli* are an important cause of diarrheal disease in both adults and children throughout the world (17). Disease syndromes range from mild traveller's diarrhea to cholera-like disease, as well as an invasive dysentery-like infection (4,13,17). The diarrheal disease caused by toxigenic *E. coli* is related to at least two virulence factors, the production of either a heat labile (LT) or heat stable (ST) enterotoxin (ENT) and the ability of the bacteria to colonize the upper small bowel (10,22,25). Both properties are mediated by bacterial plasmids (7,9,14,20,21, 23).

Little is known concerning the mode of transmission of diarrheal disease in humans, although it is presumed that enterotoxigenic strains of *E. coli* colonize the small bowel after ingestion from food or water. While toxigenic *E. coli* has been isolated from food and water, only in a few cases has the consumption of such sources been implicated epidemiologically as a vehicle for the transmission of the disease (11,15).

Recently Sack et al. (19) reported that 8% of 240 independent isolates of *E. coli* obtained from food of animal origin in the U.S. were enterotoxigenic. Strains were of diverse serotypes and did not include the classic enteropathogenic serotypes. While some of the serotypes (e.g., 0149) described are common in diarrheal disease in piglets and calves, other serotypes (e.g., 078,015) have been isolated from cases of severe diarrheal disease in humans. The authors concluded

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that "(1) the production of enterotoxin was relatively common among *E. coli* isolated from food, and (2) such foods are therefore potentially important vehicles in the transmission of diarrheal disease of humans mediated by enterotoxigenic *E. coli*." We examined these strains for antibiotic resistance as well as other plasmid mediated properties.

R PLASMIDS IN TOXIGENIC *E. coli*

Seven of the twenty strains examined were resistant to multiple antibiotics, including chloramphenicol (Cm), tetracycline (Tc), streptomycin (Sm), sulfonamide (Su), and kanamycin (Km) (Table 1). In four of the seven isolates, resistance was transferable by conjugation to *E. coli* K12 strain 711. Two strains, 79 and 173, transferred resistance at a low frequency. Strain 52 however transferred at a derepressed rate; 100% of resistant donors being able to transfer their R plasmid. Plasmid DNA was isolated by cesium chloride-ethidium bromide centrifugation (7) and characterized by agarose gel electrophoresis (12). Resistant donors contained multiple species of plasmid DNA (Figure 1, slots b,d,f) which is common in enterotoxigenic strains of *E. coli* isolated from both animals and humans (9,24). Transfer of antibiotic resistance from each strain was associated with the transfer of a single molecular species of plasmid DNA of about 50-60 Mdal (Figure 1, slots c,e,g). It is interesting to note that five of the strains described by Sack et al. (19), all isolated from hamburger in Washington, D.C., possessed the K88 surface antigen,

Table 1. R Plasmids in Enterotoxigenic E. coli

Strain[a]	*Resistance*[b]	*Transferable*	*Frequency*[c]
10	*Tc,Su*	-	
21	*Km,Su*	-	
52	*Tc,Sm,Su,Cm*	+	*1.0*
78	*Sm,Su,Cm*	+	*N.D.*
79	*Tc,Sm*	+	10^{-4}
173	*Su,Cm*	+	10^{-6}
210	*Tc,Sm,Su*	-	

[a] *From Sack et al. (19).*

[b] *Tc, tetracycline; Su, sulfonamide; Km, kanamycin; Sm, streptomycin; Cm, chloramphenicol.*

[c] *Number of resistant recipient cells/resistant donor cells.*

suggesting that they were of porcine origin. In each case K88 was associated with the ability to ferment raffinose.

PLASMIDS OF STRAIN 52

Strain 52 was examined in greater detail. It is an O149: H10 *E. coli*, a serotype commonly associated with diarrheal disease in piglets. It produces both LT and ST enterotoxins (19), is resistant to Cm, Sm, Su, and Tc, possesses the K88 surface antigen and ferments raffinose. We isolated three plasmids from this strain. A 53 Mdal derepressed R plasmid, designated pSR14 (Figure 2, slot c) confers resistance to >256, 128 and 16 μg/ml of Cm, Tc, and Sm, respectively, and belongs to the FII incompatibility group. Enterotoxin production was associated with 47 Mdal transferable plasmid, pSR15 (Figure 2, slot f) while a 45 Mdal nontransferable plasmid, pSR16 (Figure

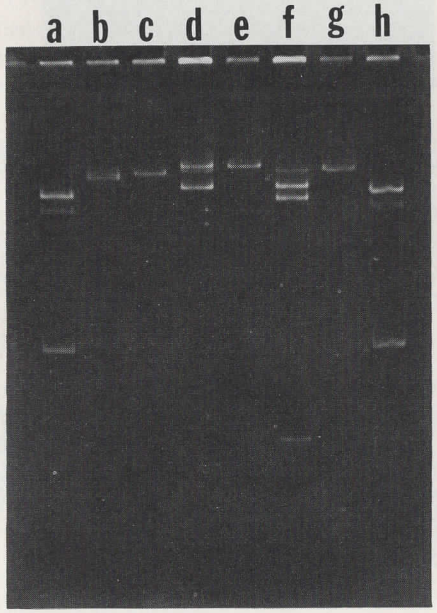

Figure 1. Agarose gel electrophoresis of purified plasmid DNA. (a) and (h) Molecular weight standards RP4 (34 Mdal), S-a (25 Mdal) and RSF1050 (5 Mdal); (b) 52; (c) 52 R$^+$ transconjugant; (d) 79; (e) 79 R$^+$ transconjugant; (f) 173; (g) 173 R$^+$ transconjugant.

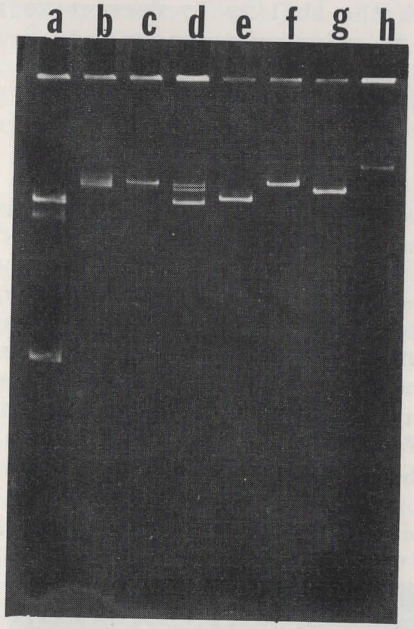

Figure 2. Agarose gel electrophoresis of purified plasmid DNA. (a) Molecular weight standards RP4 (34 Mdal), S-a (25 Mdal), and RSF1050 (5 Mdal); (b) strain 52; (c) pSR14; (d) antibiotic sensitive derivative of 52; lower band is RTF portion of pSR14 and is designated pSR17; (e) pSR17::Tn3; (f) pSR15::Tn3; (g) pSR16; (h) pSR20.

2, slot g) determined K88 production and raffinose fermentation. Both pSR15 and the resistance transfer factor (RTF) portion of pSR14 (Figure 2, slot e) were isolated by first genetically labeling the plasmids with the ampicillin transposon Tn3 (2). pSR16 was isolated by transformation (3) and selection for raffinose fermentation. Plasmid sizes were confirmed by electron microscopy.

INTERACTION BETWEEN ENT AND R PLASMIDS

The co-transfer of R and ENT plasmids from 52 was studied using the radial passive immune hemolysis assay described by Bramucci and Holmes (1) for the detection of cholera and *E. coli* LT toxins. In this assay cells were grown for 72 hr in

blood agar overlays. An additional overlay of agar containing hyperimmune burro antisera against purified cholera toxin and guinea pig complement was added. After one hr incubation hemolytic halos surround toxigenic colonies. By incorporating selective antibiotics into the medium, we could examine directly the co-transfer of ENT and R plasmids. About 1% of the transconjugants in a mating with 52 also transferred enterotoxin production. Most of the transconjugants contained multiple plasmid species. Plasmids larger than those present in strain 52 were often observed and were apparently recombinant molecules. Similar recombinational events, associated with transfer of the K88 antigen and raffinose fermentation, have been reported by Shipley et al. (20). We did isolate however an 88 Mdal plasmid (Figure 2, slot h) which transferred both resistance and enterotoxin production at the same derepressed rate as the parent R plasmid and also showed FII incompatibility. An EcoR1 restriction endonuclease digest of pSR20 (Figure 3, slot b) indicated that it contained the complete R plasmid pSR14 (Figure 3, slot a) and an additional 35 Mdal region derived from the ENT plasmid pSR15 (Figure 3, slot d). This was confirmed by DNA-DNA heteroduplex analysis. A heteroduplex between pSR20 and pSR14 (Figure 4) showed a double stranded region corresponding to the complete R plasmid, pSR14, except for a small region of about 1.5 kb at the insertion point. The remaining single stranded region derived from the ENT plasmid pSR15 can clearly be seen. This sequence also contains a hairpin loop structure with an 0.8 Kb inverted repeat flanking a sequence of about 6 Kb. The inverted repeat was also present on the parent ENT plasmid pSR15.

CONCLUSIONS

In this study we have shown that toxigenic strains of *E. coli* that were isolated from food sources contained R plasmids. In addition, we have shown as have others (6,26) that ENT and R plasmids can co-transfer and can also recombine to form a single plasmid carrying genes for both antibiotic resistance and enterotoxin production. Gyles et al. (8) recently described a single ENT-R plasmid from an *E. coli* isolated from a piglet with diarrhea. The dangers of such single plasmids carrying ENT and R genes are apparent. The problems of incompatibility between plasmids are eliminated and most importantly, while there is no direct selection for enterotoxin producing strains of *E. coli* in the environment, there is strong selection for antibiotic resistant strains, particularly on farms and in hospitals.

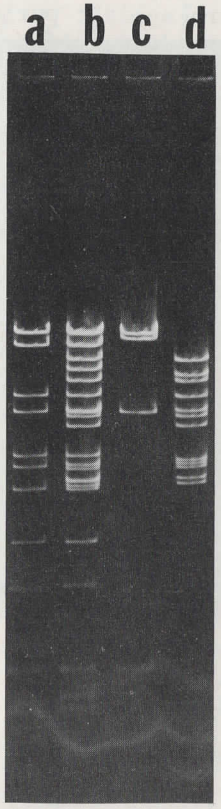

Figure 3. Agarose gel electrophoresis of EcoR1 digested plasmid DNA. (a) pSR14, (b) pSR20, (c) pSR17::Tn3 (see Figure 2), (d) pSR15::Tn3.

Up to now, toxigenic isolates from the United States and several other countries have been, for the most part, antibiotic sensitive (5,18). This has in fact led to several proposals for the prophylactic use of antibiotics to prevent diarrheal disease in travelers (5,16). We must keep in mind, however, that enterotoxin producing strains containing R plasmids are present in the environment and that the selective pressures of antibiotics may increase the prevalence of enterotoxigenic organisms.

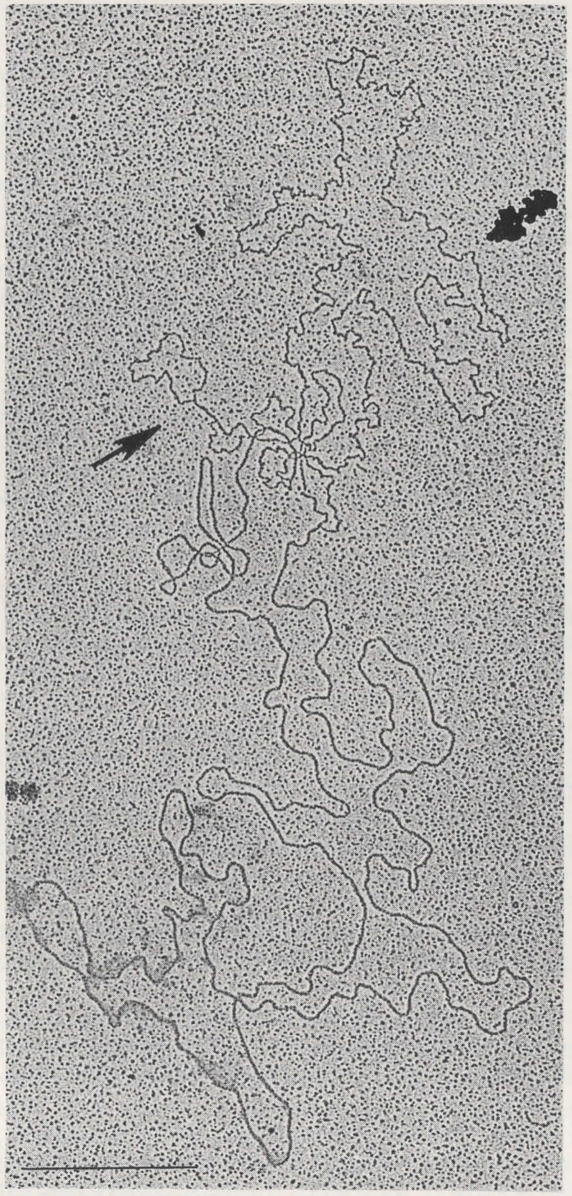

Figure 4. Electron micrograph of DNA-DNA heteroduplex between pSR14 and pSR20. Arrow indicates hairpin loop structure and inverted repeat. The bar represents 1 μm.

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DISCUSSION

Novick: Has anyone yet found a strain coming directly from animals that causes diarrhea in humans?

SILVER: That's a difficult question. Most serotypes of *E. coli* that cause a real disease in domesticated animals are different from those isolated from human cases. Very little has been done to compare the ENT plasmids from animal strains and from human strains.

Falkow: The incidence of diarrhea caused by toxigenic *E. coli* strains in the United States is very low because we have toilets. However, if serological surveys are done to see who has neutralising antibody, it's interesting to see that the one group of people in the United States with high titres against *E. coli* LT are the meat-packing workers in Iowa employed in the food industry.

SILVER: Also a high proportion of diarrheal disease goes undiagnosed. Epidemiologically, there are very few studies, such as the Crater Lake epidemic, in which the source of the organism has been pinpointed, although one would predict that the source of organisms in such diarrheal disease is either food or water.

Novick: There hasn't been a dairy product outbreak has there?

SILVER: The cheese from France was ST I think.

GENETIC MANIPULATION IN THE INSECT PATHOGEN *Bacillus thuringiensis*[1]

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Bacillus thuringiensis is a spore forming soil bacterium that is pathogenic in certain insects. During sporulation a glycoprotein is produced (3) named the δ endotoxin which is the major component of the spore coat (10). This protein is also deposited in the sporangium in the form of a crystal. In most strains the crystal is toxic to lepidopteran larvae. However, a new isolate, *B. thuringiensis* var. *israelensis* has a crystal which instead is toxic to mosquito larvae (1,2). *B. thuringiensis* is widely used in the United States on lettuce, cole crops, leafy vegetables and tobacco (12) and has also shown considerable promise in forest management to control tent caterpillars and webworms, gypsy moth, tussock moth and spruce budworm (12) either as an alternative to insecticides or in combination with them.

Despite the commercial use of *B. thuringiensis*, its genetics are almost unknown. From the later 1950's there have been reports in the literature of the irreversible loss of crystal production (6) which could indicate a plasmid-borne character. These observations were recently supported by reports that plasmid curing agents such as ethidium bromide (R.R. Azibekiyan, R.A. Belykh and Y.M. Nelyksa in Abstr. of the Third Internatl. Symp. Genet. Industr. Microorgan. 1978) and heat (13) increase the frequency of the loss of the crystal. On the other hand, Gonzales and Carlton (Abstr. Ann. Meet. of Amer. Soc. Microbiol. 1978) reported that there is no apparent correlation between crystal production and plasmids in two

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strains that they studied.

PLASMID PATTERNS

The possibility that the economically important δ endotoxin gene may be plasmid-borne led to the screening of a variety of *B. thuringiensis* strains for extrachromosomal elements. Figure 1 shows representatives of twelve serotypes of *B. thuringiensis*. The first slot contained DNA from the type strain *B. thuringiensis Berliner* which is sold as *Thuricide* (Upjohn). The second slot contained DNA from the variant *finitimus* which has no plasmids. It does produce a crystal which is not toxic. All other strains examined had at least one plasmid, but some of these could be cured without loss of crystal production. This is contrary to the plasmid-borne crystal gene hypothesis. As seen in Figure 1, apparently, as determined by molecular weight, none of the plasmids was common to two or more strains.

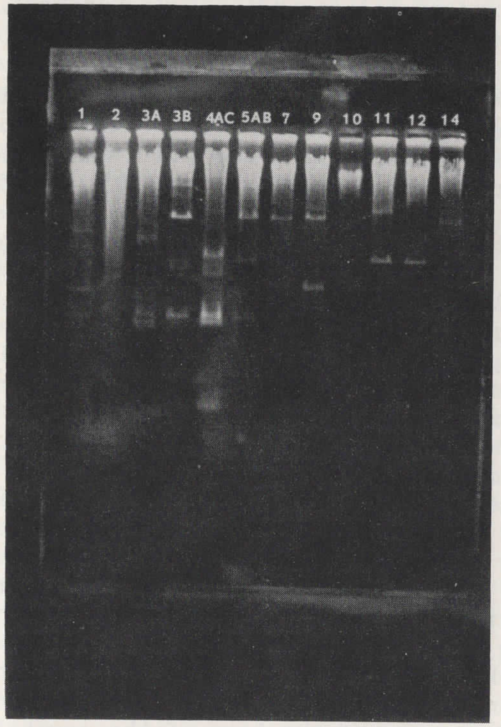

Figure 1. Representative patterns of plasmid DNA from twelve serotypes of B. thuringiensis. *Serotypes are numbered to the system of de Barjac. Cleared lysates were applied to 0.5% agarose gel and subjected to electrophoresis for 14 hr at 20v; 35 ma.*

We originally thought that plasmid patterns of different strains may be correlated with serotype. Several strains belonging to two serotypes, 1 and 3B, were compared (Figure 2). While strains of the same serotype had some plasmids in common, others were different. Slots 1 and 3 contained DNA from two different *B. thuringiensis Berliner* isolates. The plasmid patterns were identical although the strains were obtained from different sources. Slots 6 and 7 contained DNA from two *kurstaki* strains known as HD-1. The first was obtained from Howard Dulmage who first isolated it, and the second was obtained from a commercial preparation of *Dipel* (Abbott). These strains had widely different histories, yet the plasmid patterns remained essentially identical. Both could be cured of plasmids by mild heat. The eighth slot contained DNA from a derivative of the *Dipel* strain that was grown at 37°C. This strain was selected as a double mutant resistant to streptomycin and sodium azide (P. Fettig, Ph.D. thesis, The Ohio State University, Columbus, Ohio, 1976). The change in

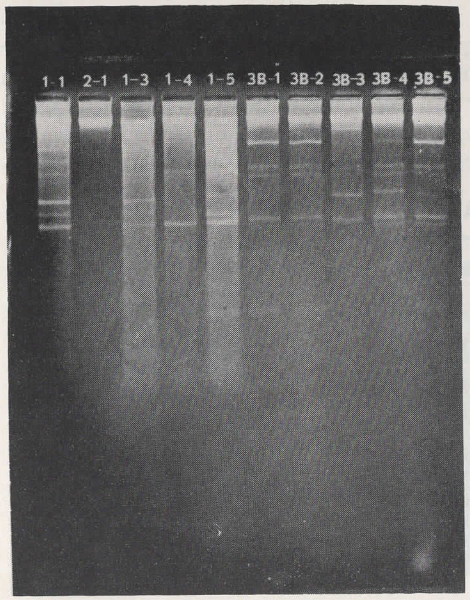

Figure 2. Comparison of plasmid DNA patterns within serotypes 1 (var thuringiensis*) and 3B (var* kurstaki*). Slot 2 contains a plasmidless control.*

plasmid pattern reflected the sensitivity of this strain to ampicillin and the apparent lack of the inducible prophage present in the parent strain.

In view of the diversity of plasmids found, we decided to work with a single strain, HD-1, which is highly toxic to many lepidopterans and is in agricultural use. This strain carries six plasmids, the molecular weights of which were determined by electron microscopy (11). In Figure 2 (slot 6), molecular weights of plasmid DNA in bands from bottom to top were 1.3, 4.9, 5.4, 9.6, 30.7, and 47.2 million daltons (Mdal) respectively. These plasmids were used as molecular weight standards.

TRANSFORMATION

A derivative of HD-1, 17A, was transformed after protoplast formation (4). Strain 17A was isolated as an ampicillin-sensitive clone following acriflaving treatment of HD-1. The sensitivity to ampicillin was correlated with the loss of the 9.64 Mdal plasmid. This strain still made a crystal and was as toxic to *Trichoplusia ni* (cabbage looper) larvae as the parent strain (5).

Protoplasts of this strain were exposed to DNA of pC194, a plasmid carrying a gene(s) for chloramphenicol resistance from *Staphylococcus aureus* (7). Transformation to chloramphenicol resistance occurred in about 10% of the bacteria which were able to regenerate their cell walls. Figure 3 shows a gel of DNA from the parent strain (3A) and four of the transformants (3B). DNA in slots 1, 2 and 4 (3B) was from stable transformants with respect to chloramphenicol resistance. pC194 was not present in an autonomous state. However, DNA in slot 3 was from an unstable transformant and did have a new plasmid slightly larger than pC194.

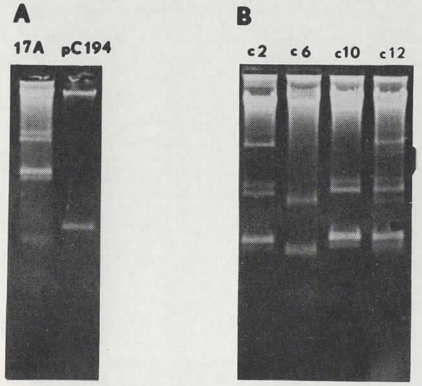

Figure 3. A: The transformable strain 17A shown with a Bacillus subtilis *strain carrying pC194. B: A sample of plasmid DNA from transformants resistant to chloramphenicol.*

We proposed that in the stable transformants pC194, acting as a transposon (7,8), had integrated into either the chromosome or one of the large plasmids. This possibility is currently being pursued through nick translation of pC194 and the Southern transfer technique to locate the putative transposon.

Since pC194 carries a gene for chloramphenicol acetyl transferase, this enzyme could be assayed in crude lysates (9) as an alternative method to show that transformation had occurred. Figure 4 shows the results of one such assay. The parent strain, 17A showed no activity whereas one of the stable transformants did show activity when assayed under the same conditions. A strain of *Bacillus subtilis* carrying pC194 as a plasmid was included as a positive control.

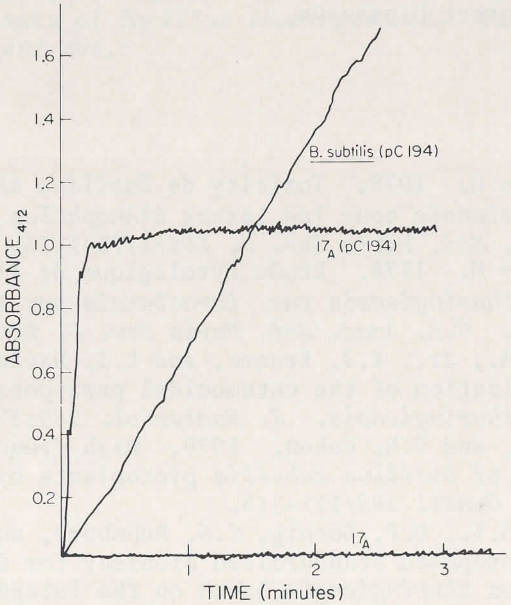

Figure 4. The change in absorbance of 5,5' dithio bis-2-nitrobenzoic acid with time measured with a recording spectrophotometer as an indication of chloramphenicol acetyltransferase activity.

FUTURE WORK

By developing a genetic transfer system in *B. thuringiensis* we hope to be able to transfer individual plasmids and show exactly which genes are plasmid-borne. An alternative explanation for some of the phenomena observed in *B. thuringiensis* might be that some of the genes are carried on transposons. The pC194 example suggests that transposition can occur. In some instances these elements may autonomous, thus providing a mechanism for irreversible loss of a trait. Transposition to the chromosome may serve to stabilize the trait.

Finally, with the wide variety of plasmids and a transformation system available the possibility of genetic engineering in *B. thuringiensis* to produce more effective insect pathogens is now foreseeable.

ACKNOWLEDGMENTS

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Part II. MAINTENANCE MECHANISMS

INVOLVEMENT OF SPECIALIZED RECOMBINATION IN THE EVOLUTION AND EXPRESSION OF BACTERIAL GENOMES

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INTRODUCTION

Intermolecular exchange of a DNA segment, that is, genetic exchange or crossing over between homologous parental chromosomes resulting in the formation of a hybrid molecule, has long been recognized (46). This marvelous process is important in providing the breadth of phenotypic diversity seen within a single plant or animal species. Classical genetics and recent studies in molecular genetics have revealed a variety of genetic exchange systems in bacteria (for reviews see 46; 68; 66). These recombination systems can be divided into two broad categories: (a) generalized, and (b) specialized. In short, *general* recombination systems mediate genetic interchange at random points between largely homologous deoxyribonucleotide segments, whereas *specialized* recombination processes act in the absence of general recombination or apparent sequence homology between the interacting DNA regions. *Specialized* recombination systems are responsible for promoting the integration, deletion, transposition, or inversion of discrete DNA segments and can also influence the expression of nearby genes (see reviews 21; 92; 59). This review is intended as a brief description of several of the more intensively studied specialized recombination systems, and relates how these processes affect both gene expression and evolution in bacteria. A more comprehensive coverage of this is also available (62).

Until recently, bacterial evolution was thought to occur by a slow process involving small chromosomal alterations

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(i.e., mutations); environmental selection for the desirable mutations; and the accumulation of beneficial mutations through intercellular genetic exchange and generalized recombination (see Figure 1a,b,c). Mutational events which involve the addition, deletion, or substitution of only one or a few nucleotides can be called *micro-evolutionary* (ref. 32 pp. 165-166; 23). Since the 1960's, however, a catalog of *macro-evolutionary* events has been amassed. These involve the reshuffling, often at relatively high frequencies, of large chromosomal DNA segments (see Figure 1d,e), and include inversion, insertion, duplication, deletion, or transposition events (Table 1), as well as the chromosomal integration of circular DNA. Micro-evolutionary alterations probably occur mainly during DNA replication or repair or both, whereas macro-evolutionary events are mediated largely by a variety of specialized

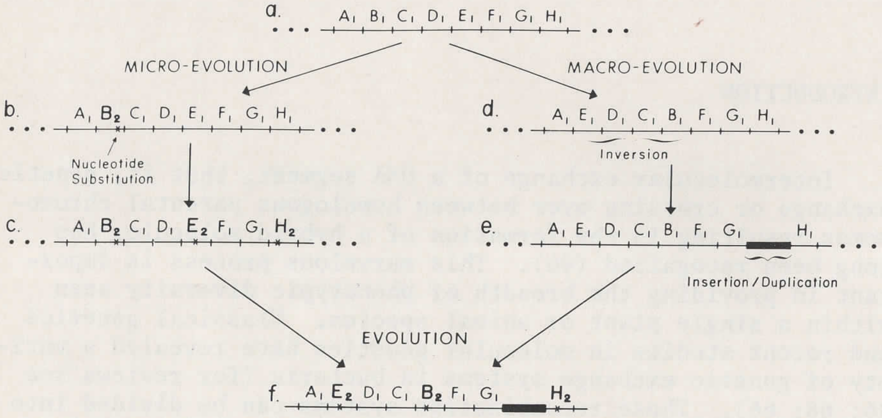
a.
A_1 B_1 C_1 D_1 E_1 F_1 G_1 H_1
MICRO-EVOLUTION
MACRO-EVOLUTION
b.
A_1 B_2 C_1 D_1 E_1 F_1 G_1 H_1
Nucleotide Substitution
d.
A_1 E_1 D_1 C_1 B_1 F_1 G_1 H_1
Inversion
c.
A_1 B_2 C_1 D_1 E_2 F_1 G_1 H_2
e.
A_1 E_1 D_1 C_1 B_1 F_1 G_1 H_1
Insertion / Duplication
EVOLUTION
f.
A_1 E_2 D_1 C_1 B_2 F_1 G_1 H_2

Figure 1. Schematic representation of evolution. The horizontal lines represent a portion of the bacterial chromosome and arbitrary genes are labelled A, B, . . . and H. The subscript, 1 or 2, after each gene designates an allelic form of that gene. The chromosomal segment shown in step (a) can undergo small single nucleotide base changes or micro-evolutionary events, such as that shown in (b) and (c). The mutated genes are indicated by a subscript 2 and pronounced lettering, and the mutations are located by an X. In addition, large chromosomal or macro-evolutionary rearrangements such as the inversion of a DNA segment (step d), the deletion of a DNA segment (not shown) or the insertion of a DNA sequence (step e) can occur. Overall bacterial evolution appears to result from the accumulation, via genetic recombination, of both micro- and macro-evolutionary chromosomal alterations, as shown in (f).

Table 1. Macro-Evolutionary Chromosomal Rearrangements

··· $A\ B\ C\ D\ E\ F\ G$ ···	*Wild-type*
··· $A\ B\underline{\ }E\ F\ G$ ···	*Deletion*
··· $A\ B\ \underline{D\ C}\ E\ F\ G$ ···	*Inversion*
··· $A\ B\ \underline{X\ Y}\ C\ D\ E\ F\ G$···	*Insertion*
··· $A\ B\ \underline{A\ B}\ C\ D\ E\ F\ G$···	*Tandem duplication*
··· $A\ B\ C\ D\ E\ F\ G\ \underline{A\ B}$···	*Duplication and transposition*

Arbitrary genes on a portion of the bacterial chromosome are labelled A, B, . . . and Y. Various macro-evolutionary DNA alterations, emphasized by underlining, are compared to a wild-type sequence shown at the top. The chromosomal integration of circular DNA, an additional macro-evolutionary event that is not illustrated here, would result in the simple linear insertion of the circular molecules into the chromosome.

events are mediated largely by a variety of specialized recombination systems. Bacterial evolution probably proceeds by the accumulation of both micro- and macro-evolutionary changes (Figure 1f). However, it should be stressed that unlike micro-evolutionary alterations, macro-evolutionary rearrangements often affect the expression of many genes and occasionally do so in an irreversible manner (e.g., sequence deletion). Hence, specialized recombination systems, which appear to contribute significantly to overall bacterial evolution, offer novel mechanisms with which bacteria can cope with the forces of natural selection.

SPECIALIZED RECOMBINATION: DEFINITION

"Genetic recombination" simply refers to all DNA exchange processes that result in hereditable altered linkage relationships of genes or parts of genes. Generalized (also known as "legitimate") recombination systems, which are encoded by most bacteria and their viruses, mediate random genetic exchange between largely homologous DNA regions; i.e., virtually any DNA segment can be exchanged, but only between DNA regions of very similar nucleotide sequence. Over the past 15 years, however, a variety of genetic exchange processes have been discovered which can function in specially constructed bacteria that are deficient in general recombination ability (e.g., *recA*-deficient *Escherichia coli* K-12). This *recA*-independent, physical joining of two apparently nonhomologous DNA segments, initially thought to be a rare, aberrant event, has previously been termed "illegitimate" recombination, to distinguish it from generalized recombination (38). However, the recent results of electron microscope and DNA sequencing studies of various macro-evolutionary events have suggested that, at least some of the responsible DNA exchange processes are functionally interrelated, a finding that appears yet to be appreciated by many. In fact, the ability to function independently of general recombination and the lack of a requirement for lengthy sequence homology between interacting DNA segments are features that appear to be shared by all of the so-called "illegitimate" recombinational events. This fact, plus the recent evidence that the responsible DNA exchange systems are highly specialized, and the indication that some of these recombination processes are functionally related now seems more sufficient to categorize these recombination systems under the term "specialized" recombination. Finally, general recombination and the various specialized recombination systems appear to be mediated by separate overall processes, but may share common components of DNA metabolism such as winding/unwinding enzymes, ligase, polymerases, various nucleases and DNA binding proteins.

SPECIALIZED RECOMBINATION SYSTEMS IN BACTERIA

Atypical recombination events in bacteria were first characterized during the study of the temperate bacterial viruses (phages). In contrast to the randomness of general recombination events, many temperate phages integrate physically as linear prophage DNA additions into one or several specific sites on the *E. coli* K-12 chromosome. Upon induction, prophage excision from the integrated state is usually precise. However, occasionally an imprecise event occurs in which only part of the prophage DNA is excised along with some adjacent bacterial DNA, giving rise to a specialized transducing phage (Figure 2; 96). Segments of the *E. coli* chromosome can also be reshuffled through duplication, deletion, inversion, and transposition (92;90). In recent years, a series of discrete DNA segments, called transposable elements, have been identified. These elements promote many of the macro-evolutionary events described above, and affect gene expression by causing insertion mutations or by carrying DNA sequences that act as genetic transcriptional promoter or termination signals, or both (92). Although seemingly different, all of the above events require little or no homology between interacting DNA regions and appear to be mediated by various specialized recombination systems. Table 2 lists some of the discrete DNA

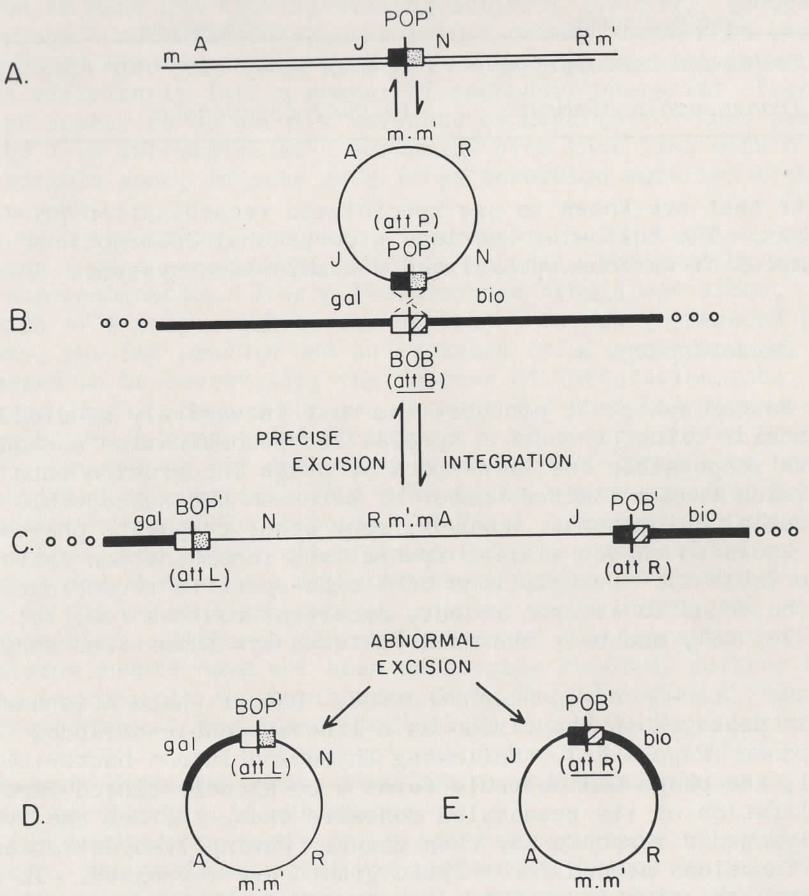

Figure 2. Integration and excision of phage λ. This unscaled diagram depicts various molecular states of the temperate bacteriophage λ. The phage chromosome is represented by a thin line(s) while the thicker lines correspond to segments of the bacterial chromosome. The rectangles descriptively labelled POP' and BOB' represent the analogous attachment sites on the phage (attP) and bacterial (attB) chromosomes, respectively. The common core sequence, O, is depicted by a vertical line in the middle of each att site whereas the unique flanking sequences P, P', B and B' are represented by the differently marked boxes. The DNA sequences marked with A, J, N, and R are phage structural genes while m·m' is the site on λ which is endonucleolytically cleaved giving rise to single-stranded, 12 nucleotide long complementary (cohesive) 5' ends, m and m'. The only bacterial genes shown are those for utilization of galactose (gal) and the synthesis of biotin (bio), which are

Table 2. Specialized Recombination Systems

Genetic unit	*Examples*
1. *Temperate bacteriophage*	*λ; Mu; ϕ434; ϕ21; P2; P22; ϕ80*
2. *Transposable element*	*IS DNA; tranposons*

units that are known to use specialized recombination processes. The following sections give general descriptions of examples of various specialized recombination systems.

A. *Bacteriophage λ*

Bacteriophage λ, probably the most intensively studied bacterial virus, encodes a specialized recombination mechanism. It is responsible for site-specific phage integration into and excision from a limited number of sites on the susceptible bacterial chromosome. However, many other temperate phages are known to encode similar specialized recombination systems (see Table 2). Descriptions of λ site-specific recombination can be found in several recent, excellent reviews (40; 16; 96; 95; 77; 78), and only the main features are summarized here.

1. *Site-Specific Recombination.* DNA of phage λ is normally packaged in the virion as a linear, double-stranded molecule (Figure 2A). Following injection into a bacterial cell, the phage DNA molecule forms a covalently closed circle by ligation of its reannealed cohesive ends. Either the lytic or lysogenic response may then ensue. During lysogenization the functions needed for λ lytic growth are repressed. In addition, the circular λ DNA molecule undergoes a site-specific reciprocal recombinational event at specific attachment or recognition sites on the phage (*attP*) and bacterial (*attB*) chromosomes, resulting in the ordered linear insertion of prophage λ into the *E. coli* chromosome where it is bounded by hybrid attachment sites (*attR* and *attL*) (67; Figure 2B,C). This event requires that the phage DNA molecule exist as a covalently closed, negatively supertwisted circle (76), and is

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situated close to the primary attB *site in* E. coli. *(A) The linear λ genome, the form in which λ DNA is packaged in the virion. In steps B through E, both λ and bacterial DNA are depicted by single lines of different width. (B) Phage circularization and lysogenization. (C) Prophage λ. (D) Circular λ*dgal. *(E) A similar λd*bio *transducing phage genome.*

catalyzed by the phage integration (*int*) protein which is known to have DNA nicking-sealing activity (77,58). Though prophage λ normally integrates into a primary *attB* site located at 17 minutes on the *E. coli* genetic map, it will integrate less efficiently into a number of secondary bacterial sites which appear to be natural variants of *attB* (87). When prophage λ is integrated at a secondary site that lies within a detectable gene, it acts as a large insertion mutation within that gene.

Upon induction, prophage λ repressor is inactivated, allowing λ gene expression. This is normally followed by excisive recombination between the prophage hybrid *att* sites. Excision of λ only requires the action of two phage-encoded products, the *int* protein and an excision (*xis*) gene product, and appears to be essentially the reverse of integration (see Figure 2B,C). Beyond the requirement for host DNA ligase and gyrase, which are needed to convert λ to a supercoiled state (76), several as yet uncharacterized *E. coli* genes have been identified by mutations that affect the λ site-specific integrative recombination process (75,78). Interestingly, these mutations also affect other specialized recombination processes and these genes may comprise a series of proteins common to DNA metabolism; namely, repair, replication, and recombination. Although the mechanisms of λ integration and excision events have not been physically defined, neither DNA, RNA, nor protein synthesis seem to be required (58). Thus, bacteriophage λ encodes a specialized recombination system that enables λ, as a discrete genetic unit, to specifically integrate into and excise from one or a limited number of sites on the *E. coli* chromosome.

In addition to mediating precise integration and excision events, the λ site-specific recombination system is able to promote recombination between two autonomous phage chromosomes. Such events are *int*-dependent and occur only at the *att* sites (94,34). Furthermore, this specialized recombination system also appears to be involved in the occasional *int*-dependent formation of certain site-specific phage deletion mutants, in which one deletion endpoint always occurs at *attP* (28).

2. *Transducing Phage Formation and Other Aberrant Recombinational Events.* Imprecise or aberrant excision of prophage λ occurs at a frequency of about 10^{-6} induced cells by a process that is independent of the prophage *att* sites; the λ *red*, *int*, or *xis* gene functions; or host general recombination ability (95). This process yields specialized transducing viruses whose genomes are composed of part of the original prophage sequences and some of the adjacent bacterial genes (see Figure 2D,E). Although the recombinational crossover

occurs mainly at random points between the interacting phage and bacterial sequences, weak evidence exists to suggest that there are preferred sites in the bacterial DNA sequences adjacent to the prophage at which abnormal excision or specialized transducing phage formation may occur (95). The formation of transducing phage particles by λ and other temperate viruses was the first example of "illegitimate" recombinational events in which neither homology between interacting DNA regions nor known general recombination systems are required.

Phage λ is also involved in several other types of aberrant recombinational events in addition to transducing phage formation (62). By examining bacterial survivors after induction of a heat-inducible λ lysogen, one can isolate bacterial deletion mutants which have lost part of the λ prophage, including λ genes involved in cell death, and some neighboring chromosomal regions. Also, vegetatively replicating λ *vir* or similar phages that express constitutive replication functions have been observed on occasion to undergo internal deletion of a large contiguous region, giving rise to λ*dv* (defective virulent) molecules (72,73). Lambda *dv* molecules lack most phage properties and exist intracellularly as multicopy circular plasmids comprising 50-250 copies/cell. Finally, because of the minimum DNA length requirements for λ packaging, many λ deletion mutants also show partial genetic duplication which allows them to be packaged. Reiteration of some of the sequences in a λ deletion mutant does occur at a relatively high frequency (10^{-4} events/cell) compared to deletion formation (10^{-7}/cell) (62). Altogether these observations indicate that λ is involved in a series of seemingly aberrant recombinational events that occur independently of site-specific (*int* or *xis*); Red-mediated; or Rec-promoted recombination, and that create specialized transducing phages and internal phage sequence duplications, as well as deletions in autonomous or integrated λ and in adjacent bacterial sequences. Recently, several Rec-independent recombination systems have been observed in *E. coli*, and could be specified by defective or noninducible prophages or prophage remnants. Such systems might mediate the aberrant genomic rearrangements observed in λ or other phages, and may play a large role in bacterial evolution. Examples of some of these specialized recombinations systems are reviewed in the following sections.

B. *The Mutator Virus, Mu*

Unlike the site-specific recombination systems exemplified by λ, temperate phage Mu can insert randomly into many chromosomal sites, often causing detectable (e.g., auxotrophic) mutations. Moreover, Mu apparently can integrate into practically any other phage, plasmid, or bacterial chromosome and can promote a variety of chromosomal reshufflings. Present knowledge suggests that the specialized recombination system of Mu represents the opposite end of the spectrum in recipient site specificity as compared to the λ phage specialized recombination system.

1. Genetic and molecular definition. Mu can integrate at many, if not all, chromosomal loci. Consequently, about 2% of the cells lysogenized by Mu concomitantly acquire a new nutritional requirement or other recognizable mutation. This represents a frequency of mutation within a single gene of 50-100 fold higher than the spontaneous frequency observed in the absence of Mu. Both genetic and physical evidence indicates that these mutagenic events occur by the *recA*-independent, linear insertion of Mu into the target gene (50).

Physical analysis of Mu phage DNA revealed several unexpected complexities. The cogent features of which (see recent reviews; 26,11,18) are summarized below and in Figure 3a. The heterogeneous terminal regions of Mu consist of seemingly random bacterial sequences that differ among phage molecules. The physical map of Mu has been divided into the α, G, and β segments, as shown in Figure 3a. Prophage immunity functions map close to one end of Mu (now termed the immunity end) and lie adjacent to the majority of known Mu genes, which are located in the 31 kilobase pair α segment. The 3.0 kilobase pair G segment frequently undergoes genetic inversion so that this region is distinguished as an internal substitution bubble in some reannealed phage molecules (Figure 3c). The remaining 1600 base pairs of actual Mu DNA, the β segment, are situated immediately adjacent to the long heterogeneous terminal sequences, referred to as the variable end (18). Therefore, disregarding the heterogeneous termini, the actual phage DNA sequences in all Mu molecules are identical except for the inverted G segment in some molecules.

2. Integration and excision. None of the heterogeneous terminal sequences associated with autonomous Mu molecules appears to be inserted during Mu integration (51,1). Furthermore, unlike cohesive-ended or terminally redundant P22, Mu molecules have no obvious physical way to circularize, as required for the chromosomal integration of many prophages (16). In addition, infecting Mu molecules apparently never form covalently-closed circles (11) and Mu integration seems to require both Mu and bacterial DNA replication (13). One could hypothesize that the infecting Mu molecule acts as a template upon which only the true Mu phage sequences are replicated, as illustrated in Figure 4. Although the mechanism is not known,

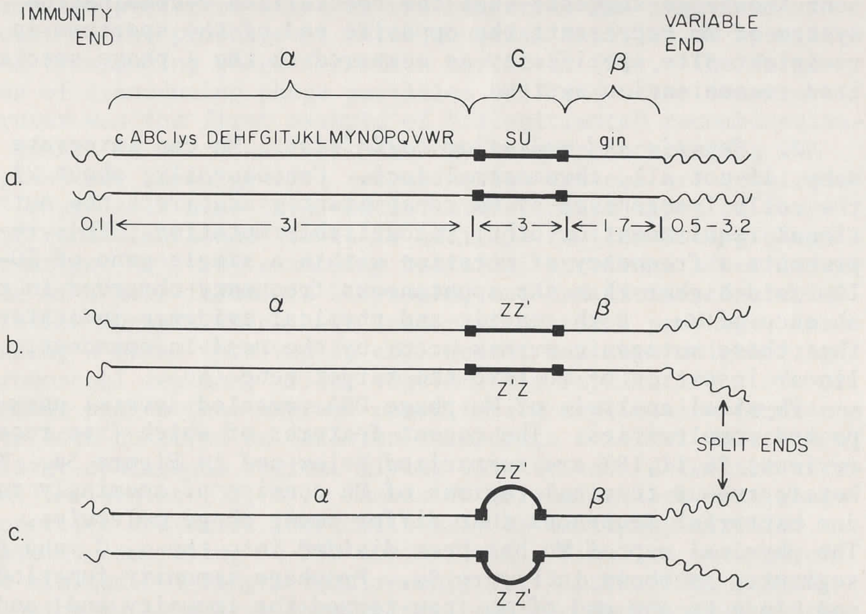

Figure 3. Physical structure of Mu molecules. Each horizontal line represents a single-strand of phage DNA. (a) Linear double-stranded mature phage DNA molecule of approximately 25 megadaltons (not drawn to scale). The heterogeneous terminal sequences are represented by a wavy line. Phage specific DNA sequences are divided into α, G, and β segments. The size is given (kilobase pairs) for each region (18,55). The invertible G segment is bounded by small (~20 base pair) inverted repeat DNA sequences (represented by solid blocks) which are thought to be involved in the inversion of this segment. The middle one-third of the G segment contains a less stable pair of inverted repeat DNA sequences, identified in the electron microscope, that are represented in steps b and c as (Z·Z'). The map order of known genes on Mu phage is shown in (a) (12; M. Howe, personal communication). The immunity gene c, *integration gene* A, *and replication gene* B, *are located at one end of Mu, termed the immunity end. Genes* S *and* U *are located in the G segment, the inversion of which is controlled by the* gin *function that is encoded in the β segment. (b,c) Double-stranded heteroduplex structure resulting from the reannealing of different denatured Mu molecules. In the molecule shown in (c), the G segment is inserted in opposite orientations in each strand, creating a substitution bubble.*

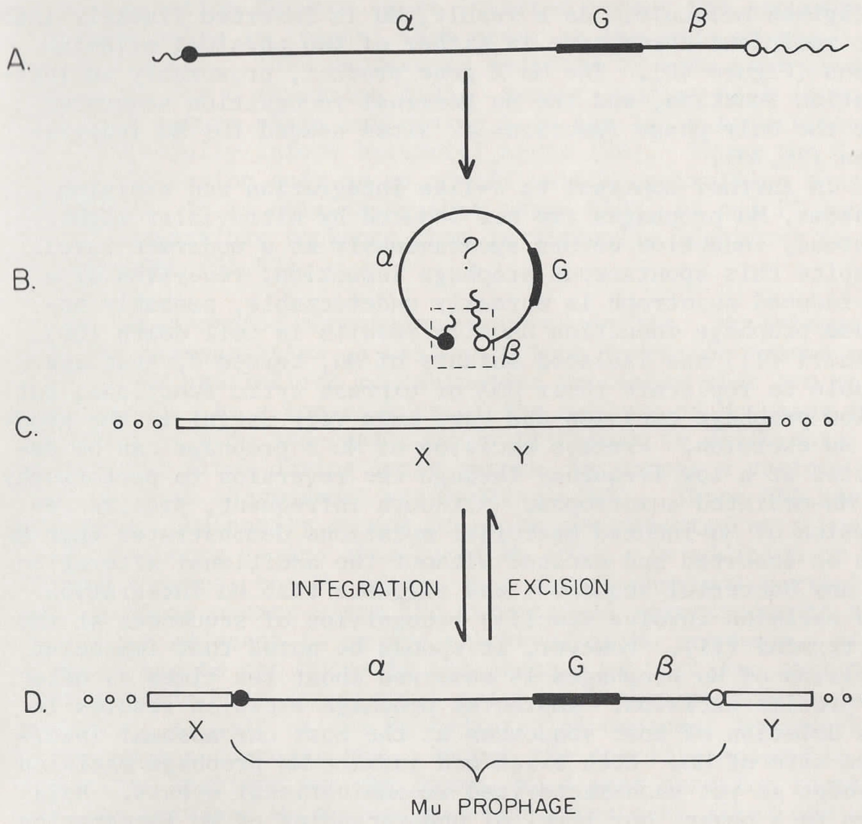

Figure 4. Hypothetical model of precise integration and excision of Mu. (A) Linear double-stranded vegetative Mu DNA. The heterogeneous bacterial DNA associated with each mature phage molecule is represented by wavy lines. Phage specific sequences α, β and G, are shown as solid lines of different width. The Mu attachment and recognition sequences are indicated by the open and filled circles. (B) Presumptive integration intermediate structure in which the true ends (i.e., att *sites) of Mu are brought within close proximity (enclosed within dashed lines), perhaps by covalent linkage or via protein. (C) Portion of a bacterial, plasmid, or phage recipient chromosome, represented by an open rectangle. (D) Physical integration of Mu resulting in the linear insertion of only the α, β and G segments of Mu into the recipient chromosome.*

Mu integration apparently involves a specific recombinational exchange between the Mu termini and the target site on the recipient molecule. As a result, Mu is inserted linearly into the recipient chromosome in either of two physical orientations (Figure 4D). The Mu *A* gene product, presumably an integration function, and the Mu terminal recognition sequences are the only phage functions or sites needed for Mu integration (36,79).

In further contrast to λ-like integration and excision systems, Mu prophages are not induced by ultraviolet light. Instead, induction occurs spontaneously at a moderate level. Despite this spontaneous prophage induction, reversion of a Mu-induced auxotroph is normally undetectable, probably because prophage induction usually results in cell death (50). Bukhari (11) has isolated mutants of Mu, termed *X*, that are unable to replicate their DNA or express lytic functions, but allow prophage excision and have been very useful in the study of Mu excision. Precise excision of Mu *X* prophage can be detected at a low frequency through the reversion to prototrophy of Mu-mediated auxotrophs. Although infrequent, precise reversion of Mu-induced bacterial mutations demonstrates that Mu can be inserted and excised without the additional alteration of any bacterial sequences and suggests that Mu integration and excision involve specific recognition of sequences at the Mu termini (11). However, it should be noted that imprecise excision of Mu prophages is observed about ten times as often as precise excision. Imprecise prophage excision results in the deletion of host sequences at the host chromosomal insertion site of Mu. Both exact and inexact Mu prophage excision involve as yet uncharacterized recombinational events. Relative to λ phage, our level of understanding of Mu integration and excision is very elementary.

3. *Prophage induction.* Current evidence suggests that prophage Mu also strikingly deviates from the behavior of other temperate phages that replicate autonomously after induction in usually not excising from the original integration site. Apparently, an induced Mu prophage generally replicates *in situ* at the original site of integration and transposes either single- or double-stranded replicas of itself to other chromosomal loci where they insert via the Mu integration system (69). The newly inserted prophage can continue the lytic replication and transposition process giving rise to multiple lysogens containing 10 or more copies of prophage Mu (13,93). Encapsulation of mature phage, cell death, and virion release complete the unique Mu lytic cycle.

4. *Internal DNA inversion regulates phage viability.* Inversion of the G segment of Mu (Figure 3C) is thought to occur by recombination between short inverted repeat DNA sequences (~20 base pairs in length) that occur at the ends of the G region (18,51). This process is mediated by a Mu gene product, termed *Gin* for *G inversion* (19). Recent studies have shown that only mature phage containing G in the lytic orientation can successfully infect bacterial hosts (56). Thus, the G segment inversion acts as an on-off switch controlling phage viability. It is noteworthy that phage D108, P1 and P7 contain an invertible sequence that is virtually identical to the Mu G segment (56,57).

5. *Mu-promoted chromosomal rearrangements.* Mu integration and transposition as well as G loop inversion events are mediated by specialized recombination processes that are encoded by Mu. As a consequence of the ability of Mu phage to replicate *in situ* and continuously transpose to different chromosomal sites during lytic growth, Mu causes a variety of aberrant host chromosomal reshufflings (62). These DNA rearrangements ordinarily do not occur or are detected at a much lower frequency in the absence of Mu (93). In addition to Mu-encoded enzymes, the Mu genome is a direct physical participant in these events since the chromosomal rearrangements always occur adjacent to or are bracketed by Mu prophage. All of these events can occur in the absence of host Rec ability. These include host chromosomal deletions, transpositions, and inversion, as well as the Mu-mediated chromosomal integration of autonomous circular DNA. Though the specific molecular mechanisms involved are not known, Mu *A* gene function together with the terminal Mu recognition and attachment sites are sufficient to promote all of these events except Mu-mediated transposition of host sequences which also requires the presumed replication function of Mu *B* gene (36,79). The simplified, hypothetical scheme illustrated in Figure 5 offers at least a visual conception of how Mu prophages might mediate various chromosomal rearrangements. However, it is more likely that these events occur by the scheme proposed by Faelen and Toussaint (35) and expanded in a recent review (62). Although Mu-mediated chromosomal alterations can occur independently of host general recombination, specific DNA segments like Mu phage or transposons (see later section) which are recognized and exchanged by specialized recombination enzymes can also be substrates for generalized recombination systems, as noted in Figure 5. In fact, it seems likely that generalized recombination between similar mobile elements (e.g., Mu or transposons) contributes significantly to the evolution of bacteria in nature. Therefore, besides promoting phage integration and transposition events, the Mu-mediated specialized

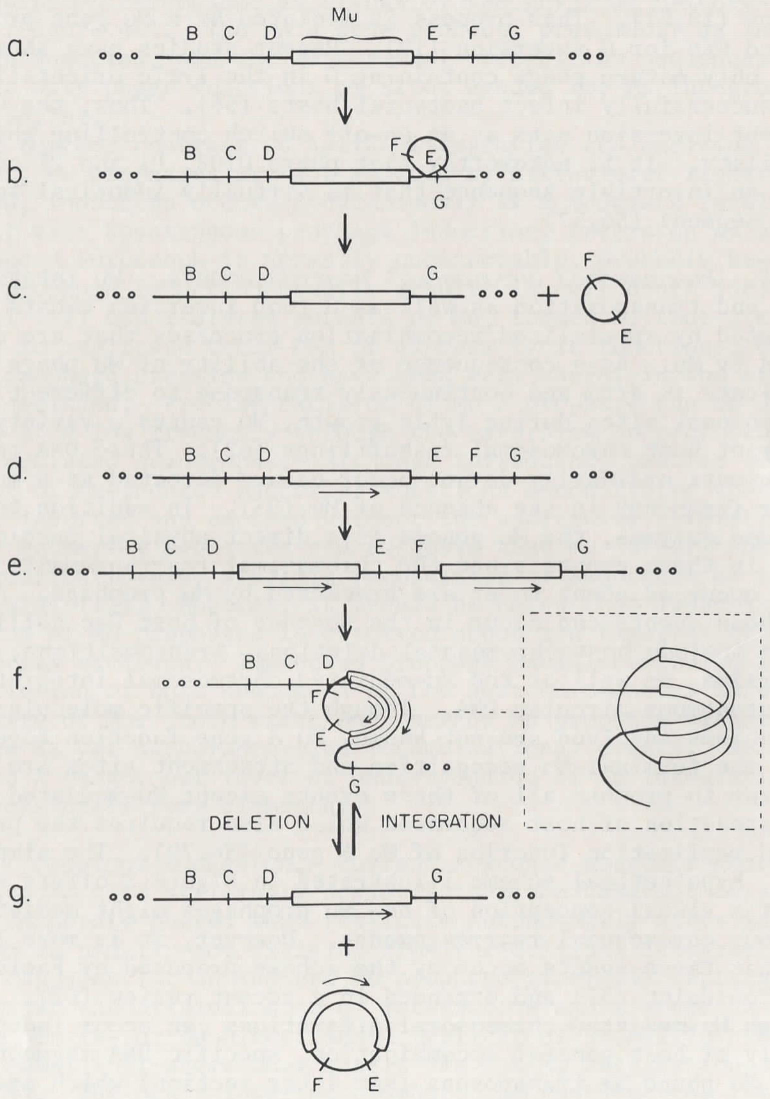

Figure 5. Simplified hypothetical scheme for Mu-promoted deletion of host chromosomal DNA. (a) The thin black horizontal line represents a portion of a double-stranded bacterial chromosome which contains a Mu prophage, depicted by the open rectangle. Several hypothetical host genes are denoted alphabetically and the Mu prophage is inserted between host genes D and E. Many Mu-promoted host DNA deletions apparently occur from either Mu prophage terminus to random points within the adjacent chromosomal DNA and the prophage always remains intact. These Mu-mediated deletions might result from a recombinational exchange between either Mu terminus and nearby host sequences. (b) Deletion of host genes E and F could result from a reciprocal genetic exchange between a Mu terminus and a chromosomal site situated between genes F and G. (c) The resulting chromosome would contain a deletion of some host sequences immediately adjacent to one prophage terminus. The deleted material might exist as a nonreplicating small circle which would be diluted out of the cell population during growth. Although this scheme indicates how Mu might mediate deletion formation, a more sophisticated model (62) is a more probable mechanism for this reaction.

It is important to note that although the above events do not require participation of the host Rec system, deletion formation can nevertheless occur between nearby Mu prophage via host chromosome-mediated general recombination. (d,e) Partial induction of a Mu monolysogen could result in the formation of a dilysogen. (f) Recombinational crossover between the nearby prophages could occur at any point along the paired Mu genomes. (g) In this example, general recombination between the identically-oriented, paired, nearby prophage results in the same chromosomal product as shown in (c). However, the deleted material now consists of all of the host DNA sequences initially located between the prophages (i.e., host genes E and F) as well as one entire Mu genome.

As shown in diagrams (g) to (f), the Rec-dependent integration of circular DNA into the chromosome can occur if both circular DNA's contain a Mu prophage. Though not shown, deleted host sequences like those depicted in step (g) could be reinserted at a new chromosomal prophage locus, an event that would constitute site-specific, Rec-dependent translocation of host DNA. Thus, Rec-dependent integration might occur by the apparent reversal of the deletion process and Rec-dependent transposition encompasses a deletion event followed by reintegration of the deleted sequences at a new chromosomal locus.

recombination system(s) is involved in mediating macro-evolutionary chromosomal alterations (62). In addition, Mu phage can undergo internal deletions entirely within the phage genome, but this process remains largely uncharacterized (19, 36).

6. *Effect of Mu on bacterial evolution.* The host ranges of temperate phages are usually limited to one or two genera

because other bacteria lack the proper cell surface receptors or the phage is unable to replicate once injected intracellularly, or both. Mu phage is unusual in that it can infect bacteria in at least 5 different genera. In addition, when introduced into cells via conjugationally promiscuous plasmids, Mu has been observed to replicate, lysogenize, and promote chromosomal rearrangements in many diverse Gram negative bacteria which are not normally susceptible to Mu infection (30). Mu specialized recombination systems mediate a variety of host genomic rearrangements, some of which occur relatively frequently. For instance, the frequency of Mu-induced transposition of any specific chromosomal gene can be as high as 2×10^{-4} events per induced Mu lysogen (35). Assuming that the *E. coli* genome consists of about 3000 genes, then in an induced population of Mu lysogens the phenomenally high frequency of 50% of cells should contain a transposed DNA sequence. Also, transposition of large, complex DNA segments to other sites can place them under different genetic regulatory controls or, if transposed to a plasmid vector, can promote rapid intercellular dissemination of hereditary information. Therefore, Mu and λ may represent opposite ends of a spectrum of specialized recombination systems, each of which recognizes a specific class of elements (e.g., λ, Mu, or insertion sequence elements) and which can effect their specific insertion into only one, a few, or unlimited recipient DNA sites. Many of the properties of Mu have been reviewed in detail elsewhere (11,12,26,50,62).

Recently, an increasingly large group of transposable genetic entities has been detected in many diverse bacterial genera. These elements do not appear to replicate in a physically autonomous state like a plasmid and cannot exist extracellularly like a virus, but they can promote a variety of host genetic alterations. As described below, these mobile units appear to be functionally related to λ and Mu viruses in that all are recognized as discrete units by specialized recombination processes.

C. *Transposable Genetic Elements*

1. *Definition.* Transposable genetic elements are discrete mobile DNA segments that apparently are common constituents of plasmid, virus, and bacterial chromosomes. These elements were intially detected by their unusual genetic behaviour, namely, their ability to transpose self-encoded phenotypic traits from one replicon to another, or to transpose to a known gene and inactivate it. Beyond their simple transposition to a chromosomal locus, these remarkable elements cause a variety of macro-evolutionary chromosomal rearrangements similar to those promoted by Mu phage. Furthermore, some transposable DNA units encode sequences that behave as transcriptional start and stop signals, and can act as supernumerary regulatory switches affecting gene expression (see reviews 22,59,90,91).

Transposable elements have been divided into two classes, both for historical and structural reasons. They range in size from about 750 to greater than 50,000 nucleotide base pairs and are structurally defined by repeated DNA sequences at their termini (Figure 6). The small insertion sequence (IS) elements, representing the first class, were discovered in the late 1960's as unusual insertion mutations; they do not encode any known phenotypic traits. The second group consists of relatively large mobile units that encode phenotypically identifiable traits such as antibiotic resistance or toxin production, and were physically identified in the mid-1970's. These large elements, seemingly more complex than IS units, consist of a central sequence bracketed by direct or inverted DNA sequence repeats which sometimes comprise a bona fide IS element (Figure 6). Although this classification scheme is tenuous, it does emphasize the hierarchy of structural

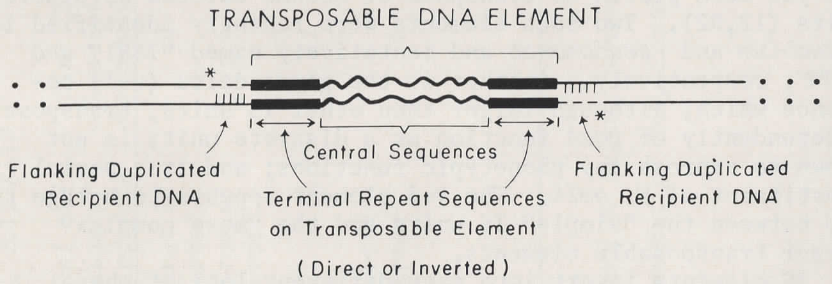
TRANSPOSABLE DNA ELEMENT
*
*
Central Sequences
Flanking Duplicated Recipient DNA
Terminal Repeat Sequences on Transposable Element
(Direct or Inverted)
Flanking Duplicated Recipient DNA

Figure 6. Diagram of the structure of a transposable element. In this unscaled diagram a transposable DNA element (delineated by the rectangles and wavy line) is inserted within a recipient DNA sequence (depicted by the thin continuous or broken lines). Both IS and Tn units include a central DNA segment (wavy line) flanked by terminal sequences (rectangles) that are repeated either in direct or inverted order. During insertion, staggered single-strand cleavage apparently occurs at the sites marked by asterisks, and the extended single-strand ends of the recipient molecule are joined to the transposable element. Subsequent gap filling DNA synthesis (broken lines) and ligation creates duplicated recipient molecular sequences at the ends of the inserted Tn or IS element.

complexity in transposable elements. Regardless of complexity, however, all of these discrete DNA units promote chromosomal rearrangements in bacteria. No transposable element has yet been shown to replicate or exist in a physically autonomous state; they are always linearly inserted within a DNA molecule.

2. *IS Elements*.

a. General properties. The study of the bacterial chromosome via mutation has revealed a variety of novel, strongly polar, revertible mutations. Intensive genetic and molecular analyses showed that these mutations are caused by the chromosomal insertion of several small discrete DNA units, now known as IS elements (91,92). The general properties of IS elements are summarized below and in Table 3. The physical aspects of IS elements, which have been deduced from recent DNA sequence analyses (62) are discussed in more detail with the larger transposable elements. At present, there are five named IS elements, IS1 through IS5, which range from 750 to 1400 nucleotide base pairs in length. In addition, an assortment of unclassified "insertion-like" elements have been identified in *E. coli* and many other bacterial genera, but have not yet been proven to transpose or become deleted as discrete units (12,92). Two such elements were recently identified in *Rhizobium* and *Pseudomonas* and tentatively named "ISR1" and "ISP1", respectively. Another is the gamma-delta (γ-δ) sequence which, although larger than other IS units, transposes independently of *recA* function as a discrete unit; is not known to express any phenotypic functions; and is a normal constituent of *E. coli*. The γ-δ element appears to bridge the gap between the "simple" IS units and the "more complex" larger transposable elements.

IS elements insert into many different loci of phage, plasmid, or bacterial chromosomes (3), but certain genomic regions appear to be preferred areas for insertion. It appears that IS element integration is neither entirely random like Mu integration, nor as specific as the integration of λ. Rather, IS units often express what has been termed "regional" specificity; that is, the ability to integrate as a discrete unit at many sites within a preferred short recipient DNA segment.

First discovered in *E. coli*, one or more classes of the bona fide IS elements have now been found in the related genera *Salmonella*, *Citrobacter*, and *Shigella*, on the phage and plasmids of enteric bacteria, and in the Gram positive species *Bacillus subtilis* (84). Owing to the conjugational promiscuity of various plasmids, one would expect a wide dissemination of plasmid-borne transposable elements. In fact, IS-like

Table 3. IS Elements in E. coli *K-12*

Element	*Size (bp)*	*Size (bp) and orientation of terminal repeats*	*Size (bp) of recipient duplication*[a]	*Copies/genome*[b]	*Total DNA (bp)*	*References*
IS1	768	28 of first 34 inverted	9	8	6200	11,42,81,92
IS2	1327	32 of first 41 inverted	5	5	6650	11,29,39
IS3	~1200	?	?	≥3	3600	11
IS4	1400	?	11	≥1	1400	11,45
IS5	1250	50 inverted	?	≥1	1250	11,20
γ δ	~5800	35, inverted[c]	5	≥4	23000	10,44
					42100 bp 1% of genome	

---

[a] *Sequences abutting IS termini.*

[b] *From references 80,17,31,92, and M. Guyer, personal communication.*

[c] *Terminal 28 bp identical to termini of Tn1, 2 and 3 (i.e., TnA).*

units have been detected physically or genetically, or by both means, in several widely varied bacterial genera including *Proteus, Pseudomonas, Rhizobium, Staphylococcus, Streptococcus* and *Streptomyces* (62). Thus, although the data are limited, these elements appear to be widely distributed among prokaryotes.

DNA hybridization studies revealed that multiple copies of various IS elements exist as normal constituents of the *E. coli* K-12 chromosome (Table 3). For this reason, it has been impossible to determine the exact transposition frequency for any specific IS element. Moreover, the transposition frequency almost certainly varies with the specific recipient site involved. However, examination of spontaneously occurring mutations in *E. coli* or phage showed that IS element insertion alone can account for 10-15% of all mutations within specific genes (91). As mentioned above, IS-induced mutations are revertible. The function of a mutated gene carrying an IS element can be restored at frequencies of 10^{-3}-10^{-8}/cell depending on the IS element, its orientation, and the gene involved (92). Thus, precise excision of IS elements, known to be *recA*-independent, is probably also mediated by a specialized recombination system.

b. Abnormal genomic rearrangements. Insertion and precise excision of IS elements appear to involve enzyme recognition of specific sequences at the IS element termini, though none of the enzymes involved has been isolated. In addition, specialized recombination events involving these same IS element termini are seemingly responsible for various chromosomal alterations. For instance, considerable data indicate that at least some IS elements generate chromosomal deletions ranging in size from several hundred to as large as 20,000 nucleotide pairs in length. These deletions generally terminate at either end of the integrated IS element and extend outward to non-randomly distributed endpoints within the adjacent bacterial sequences, leaving the IS element intact (Figure 7). Following a primary deletion event the intact IS element can transpose or cause secondary and tertiary deletions. Although corroborative data are presently scarce, it appears likely that IS elements mediate a variety of macro-evolutionary chromosomal rearrangements.

c. Regulation of gene expression. Current evidence suggests that IS elements encode DNA sequences that can act as transcriptional start or stop signals, though the mechanisms of these effects are not well understood (92). Depending upon the orientation of an inserted IS element, the function of the mutated gene alone may be abolished or, in addition, the expression of promoter-distal genes in an operon can be either

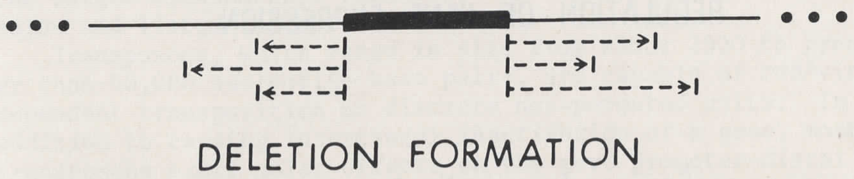

Figure 7. Deletion formation mediated by transposable elements. The solid horizontal line represents a portion of a chromosome containing an inserted transposable element, depicted by the solid rectangle. The sequences lost in a hypothetical deletion event are each represented by a dashed line.

eliminated or enhanced (62).

Since transcription occurs unidirectionally from a promoter sequence, only one strand of an inserted IS element is transcribed along with the genes of an operon. IS*1* and IS*4* exert polar effects when inserted in either orientation within an operon and these elements apparently encode transcriptional stop signals in both DNA strands. In contrast, IS*2* eliminates promoter-distal gene expression only when the IS element is in one orientation (I). In the opposite orientation (II), IS*2* can act as a transcriptional promoter. For example, when inserted in orientation II within the galactose (*gal*) operon control region of *E. coli*, IS*2* increased *gal* gene expression three-fold above expression of the normal *gal* operon without an insertion element. Thus, gene expression in an operon can be regulated by the insertion of a DNA segment that, like IS*2*, encodes both transcriptional start and stop signals, as illustrated in Figure 8 for the *lac* operon. IS*2* mediated control mimics Mu G segment inversion which controls phage viability, as discussed earlier. Present data indicated that a promoter located outside the G1 segment is responsible for initiating transcription of essential genes located within the G segment. When the G1 segment is positioned in the nonviable orientation, the essential G1 segment genes are not expressed and nonviable phage are produced. Recently inversion of an 800 base pair IS-like DNA segment that behaves like IS*2* was shown to control the alternate expression (phase variation) of flagellar antigens in *Salmonella* (88,97). Therefore, in addition to causing insertion mutations, IS elements can effect the expression of adjacent bacterial genes. Though only a few examples exist, the regulation of gene expression by genetic inversion seems to be an established process, at least in bacteria and their viruses.

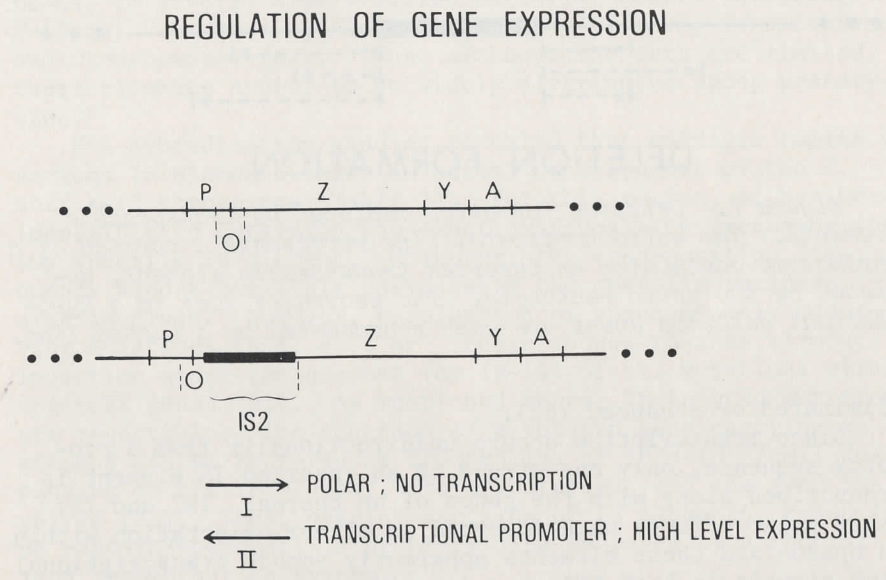
REGULATION OF GENE EXPRESSION
P
Z
Y
A
O
P
Z
Y
A
O
IS2
POLAR ; NO TRANSCRIPTION
I
TRANSCRIPTIONAL PROMOTER ; HIGH LEVEL EXPRESSION
II

Figure 8. Regulation of gene expression. The lactose operon, consisting of the promoter (P) and operator (O) regions plus the structural genes Z, Y, and A for beta-galactosidase, beta-galactoside permease, and transacetylase, respectively, is depicted by the top horizontal line. The lactose operon depicted in the bottom line is interrupted by the IS2 element inserted within the operator gene. In orientation I, this IS2 element prevents operon transcription and promoter-distal gene expression. The same element in orientation II causes high level expression of the lactose genes. The arrows simply refer to the orientation of the IS element.

3. Phenotypically Identifiable Transposable Elements: General Properties and Host Range. Genetic evidence, amassed during the 1960's, indicated that the plasmid DNA segments encoding resistance to one or more medically useful antibiotics could recombine with phage, bacterial, or other plasmid chromosomes. Subsequently, with the development during the early 1970's of techniques to isolate and physically analyze plasmid DNA, a variety of large discrete transposable DNA segments that specify various phenotypically recognizable traits were identified (see reviews 21,22,62; and Table 4). In the initial description of a large transposable element (Tn*1*) that encodes β-lactamase production (i.e., penicillin resistance phenotype), Hedges and Jacob (47) proposed the term *transposon* (abbreviated Tn) to define specific DNA sequences with transposition potential. This term is now commonly applied to the larger transposable elements, some of which are discussed below and listed in Table 4.

Transposons, which range in size from about 2000 to greater than 50,000 nucleotide base pairs, are capable of *recA*-independent transposition as discrete non-permuted units. In addition to causing insertional inactivation of a gene, most transposons exert polar effects on the more promoter-distal genes in an operon (59). Moreover, like the IS units, these larger transposable elements are responsible for promoting many different types of chromosomal rearrangements as depicted in Figures 7 and 9. Though the first transposons to be

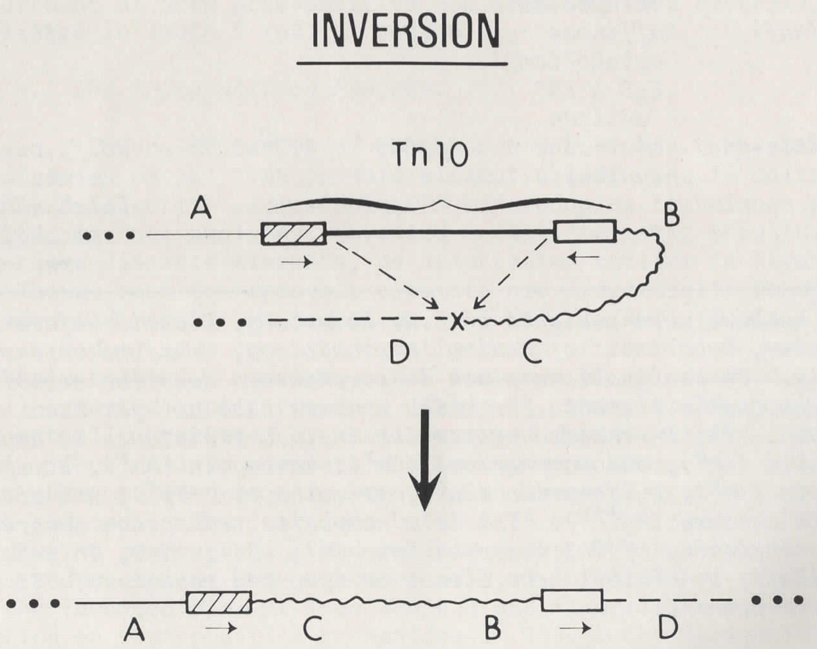

Figure 9. Transposon-promoted inversions during imprecise Tn unit excision. The Tn10 transposon is shown here inserted within a chromosome which is represented by solid, wavy, and broken lines. Chromosomal regions A through D are labelled. The 1400 bp repeats on Tn10 are shown as rectangles and their orientation is indicated by the arrows below each. The inside end of each 1400 bp repeat may recombine (see dashed arrows) with some adjacent sequence, denoted here by an X. This recombination event results in the loss of the Tn10 sequences normally contained within the terminal 1400 bp repeat sequences, as well as the inversion of one 1400 bp repeat and some adjacent chromosomal material (see ref. 83 for details).

Table 4. Some Phenotypically Identifiable Transposons

Tn element designation	*Tn encoded properties*	*Total Tn length (bp)*	*Transposition frequency (events/cell)*	*References*
*Tn*1	Ap^r	4,600	10^{-2}	47,49
*Tn*4	Ap^r, Sm^r, Su^r Hg^{++r}	20,500	10^{-6}-10^{-7}	63,64
*Tn*5	Km^r	5,200	10^{-3}-10^{-2}	8,9
*Tn*7	Tp^r, Sm^r	12,750	5 x 10^{-4}	5,6
*Tn*9	Cm^r	2,500		61,41,70
*Tn*1681	*heat stable enterotoxin*	2,060		89
*Tn*951	*lactose catabolism*	16,600		24,25
Tn(Raf)	*Raffinose catabolism* H_2S *& K88 antigen*	40,000		85
Tn(his-gnd)	*histidine synthesis*	44,000	10^{-2}-10^{-4}	*Wolf, pers. comm.* *Palchaudhuri et al., pers. comm.*

Tn numbers were assigned by E.M. Lederberg, Plasmid Reference Center, Department of Medical Microbiology, Stanford University. Tn designations given in parentheses describe certain transposable elements for which numbers have not yet been given. The Tn-encoded properties include resistance to ampicillin (Ap^r)*, chloramphenicol* (Cm^r)*, kanamycin* (Km^r)*, streptomycin* (Sm^r)*, sulfonamide* (Su^r)*, trimethoprim* (Tp^r)*, and divalent mercury* (Hg^{++r})*. Tn4 is a composite transposon apparently consisting of Tn3 inserted into Tn21 (64). Tn9, Tn(R-det), Tn(Raf), and Tn1681 have direct or inverted repeats of IS1 at their termini.*

characterized all encoded enzymes responsible for antibiotic resistance, later ones were shown to specify resistance to heavy metal ions (e.g., Hg^{++}) or enzymes involved in the metabolism of lactose, raffinose, toluene, xylene, or salicylate (Table 4 and ref. 62). Furthermore, the genes for enterotoxin production (89) and the genes for the synthesis of the K88 bacterial surface antigen that is responsible for intestinal colonization of animals (85), previously identified on plasmids, have now been shown to exist as transposons. Finally, a discrete segment, carrying the histidine genes, of the *E. coli* K-12 chromosome was recently observed to transpose at a relatively high frequency (Table 4). Transposons have now been identified on the plasmids or host chromosomes of a large assortment of both Gram-positive and Gram-negative bacteria, as listed in Table 5 (62).

Table 5. Bacterial Genera in which Transposons have been Identified

Citrobacter
Escherichia
Haemophilus
Klebsiella
Proteus
Pseudomonas
Rhizobium
Salmonella
Serratia
Shigella
Staphylococcus
Streptococcus
Yersinia

4. *The Transposition Process.*

a. Molecular nature of transposable elements. Physical examination of all transposable elements, including IS units, mainly by electron microscopic DNA heteroduplex techniques and by DNA sequence analyses, revealed a characteristic structure for these discrete elements, as illustrated earlier in Figure 6. Though most transposable elements are structurally bounded by inverted repeat DNA sequences, the Tn*9*, Tn(Raf) (Table 4), and Tn(R-det) (62) contain direct repeats of the IS1 sequence at their termini. However, since the ends of IS1 contain a small inverted repeat sequence (Table 3), these transposons are actually bracketed by small inverted repeats at their termini. Thus, all IS elements and transposons whose ends have been characterized to date, contain an inverted terminal repeat sequence. The junctions between IS or Tn elements (including Mu prophage [2]) and recipient host DNA have directly repeated recipient DNA sequences of 5, 9, or 11 bp at each end of the inserted element (see Table 3 and Figure 6, and next section on transposition mechanisms). Though the nucleotide composition of the repeated recipient sequence varies from one insertion site to another for a particular element, each transposable element is always associated with a repeated recipient DNA sequence of specific length as shown in Table 3 (14,39,42, 54,62).

The large inverted repeat termini located on many transposons make these elements easily identifiable in the electron microscope, as illustrated in Figure 10. In addition to forming characteristic stem-loop structures of constant size, insertions of any transposon will increase the size of the recipient molecule by a discrete length. Further evidence for a specific transposition event can be obtained by extensive

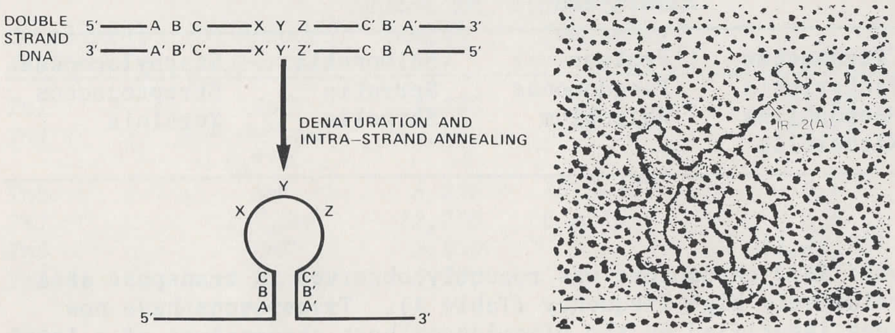
DOUBLE STRAND DNA
5' A B C X Y Z C' B' A' 3'
3' A' B' C' X' Y' Z' C B A 5'
DENATURATION AND INTRA-STRAND ANNEALING
Y
X
Z
C B A
C' B' A'
5'
3'
IR-2(A)

Figure 10. Characteristic intra-strand structure of transposons showing inversely repeated termini. A double-stranded DNA segment containing a transposon with complementary termini is depicted at the top left. Following denaturation and intra-strand annealing, the complementary termini of the transposon reanneal to form a short double-stranded stalk or stem. The central sequences of the Tn unit (XYZ) are enclosed in the single-strand loop at the top of the stem. The electron micrograph shows a self-annealed single-strand of a small plasmid containing the Tn601/Tn903 element. The double-strand stem (labelled IR-2) represents the 1000 base pair inverted repeat sequences at the termini of this element.

restriction endonuclease analysis of several molecular isolates containing the same putative Tn element, since Tn units transpose as non-permuted DNA segments and consequently retain the same restriction sites (62).

b. Mechanism of transposition. Transposition of transposons occurs at frequencies which range from 10^{-7}-10^{-1} events per cell (Table 4) (62) and probably depend upon the bacterial host, the recipient chromosomal sites, and the Tn element itself. Transposition of discrete, non-permuted units implies that specific sequences at the ends of Tn elements are enzymatically recognized during insertion. In fact, genetic studies of mutant transposons have demonstrated that the inversely repeated sequences at the Tn termini are necessary for transposition (48,74). Furthermore, recent DNA sequence analyses of various inserted transposons or IS elements have verified that transposable elements are transposed as discrete units (14,42,60,81). Like Mu phage, some transposons (e.g., Tn*5* and Tn*10*) appear to insert into randomly assorted chromosomal loci. However, most other transposon insertions occur predominantly at preferred areas of the recipient chromosome (a phenomenon

termed "regional" or "local" specificity). Although the recipient chromosomal recognition sequences for Mu and some transposons may be short (2-3 bp) and randomly distributed, the recipient recognition sequences for most transposons appear to be complex. The point of cleavage of recipient DNA during transposition (the Tn insertion site) probably occurs at variable distances (up to 500-1000 bp) from the recognition site in different insertion events; hence the "regional" specificity for Tn insertions. Type I restriction endonucleases which exhibit this pattern of behaviour, or similar enzymes, may be responsible for the observed preferred areas of Tn insertion.

Perhaps the most unusual aspect of the transposition of Mu, IS, or Tn elements is that movement to a new site is not accompanied by loss of the element from the original locus (7, 86). This implies that an obligatory and integrally linked replication and transposition process, similar to that already discussed for Mu, occurs with other transposable elements. The transposition process, diagrammatically depicted in Figure 11, results in the linear insertion within the recipient chromosome of the transposable element bracketed by a direct duplication of 5, 9, or 11 bp of recipient DNA at the inserion site. The recipient DNA repeat sequences bracketing any specific element (e.g., IS1) always have the same length (e.g., 9 bp), but vary in nucleotide composition at different insertion sites (14,42,81). Although the mechanism and requirements of transposition are unknown, data obtained recently showed that some of the larger transposable elements (Tn*3* and Tn*5*) encode one or more proteins or have sites in addition to the Tn termini that are necessary for transposition (33,74,48). Based on the similar structural consequences of transposition of Mu, Tn, or IS elements, several models have been proposed to explain this process. Grindley and Sherratt (43) recently outlined a model for transposition by single-strand DNA exchange. Another model, proposed by Shapiro (86, and this volume), involves temporary fusion of the donor and recipient molecules, each carrying a copy of the transposable element. Both models accommodate transposition without deletion of the donor transposable element, but the latter model allows for transposon-mediated plasmid cointegration, an event which has been observed during transposition of Mu as well as some transposons.

Reversion of Tn-induced mutations by precise excision of the Tn element occurs independently of *recA* function and at frequencies far below the normal Tn transposition frequency. Also, since precise excision of a Tn element would involve not only removal of the inserted Tn unit, but also of one of the 5, 9, or 11 bp flanking repeat sequences, it is likely that precise excision of Mu, Tn, or IS units occurs by a

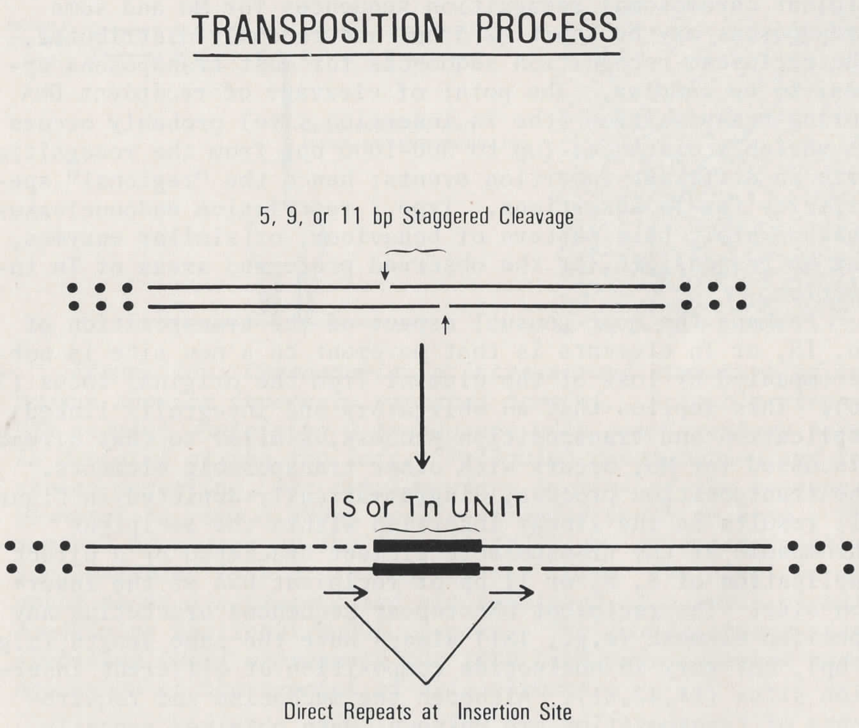
TRANSPOSITION PROCESS
5, 9, or 11 bp Staggered Cleavage
IS or Tn UNIT
Direct Repeats at Insertion Site

Figure 11. Transposition process. The horizontal lines represent a portion of the double-stranded recipient chromosome. The transposable element is depicted by a filled rectangle. Apparently, during Tn, IS, or Mu phage insertion, an enzyme(s) creates a single-strand cleavage (marked by small arrows), staggered by 5, 9, or 11 bp in each recipient DNA strand. During the transposition process the extended single-strand ends of the recipient molecule are joined to the transposable element, with the complementary recipient sequences on each strand being newly synthesized (represented by dashed lines), as illustrated in the bottom diagram. As a result of this process, a 5, 9, or 11 bp sequence of recipient DNA at the insertion site is repeated in direct order on both sides of the inserted element. Precise excision of the transposable element, an event which apparently differs from transposition, results in the removal of not only the transposable element, but also one of the directly repeated recipient sequences.

process separate from transposition. Two unusual *recA*-independent recombination pathways in *E. coli* K-12 have recently been analyzed, and perhaps these or similar bacterial processes may be responsible for precise excision of transposable elements (37,53).

EFFECT OF TRANSPOSABLE ELEMENTS AND VIRUSES ON BACTERIAL EVOLUTION

It seems reasonable to suggest that, like temperate phages, transposable genetic elements exist universally in bacteria. In addition to causing insertion mutations when inserted within a chromosome, both temperate phages and transposable elements can mediate various macro-evolutionary chromosomal rearrangements and some of these elements are involved in the regulation of gene expression. Specific examples of how transposable elements and temperate phages affect bacterial evolution have been given in the previous sections and have been extensively reviewed (12,62,91,92). Although the information presented in this brief review is confined to bacteria, it is important to note that current genetic and molecular evidence indicates that similar transposable elements are an important factor in gene expression and evolution of higher organisms (15). Several additional points can be discussed.

A. Evolution of Transposable Elements

Based on size and genetic complexity, there appear to be at least three levels in the hierarchy of transposable elements. The simplest transposable units seem to be the IS segments which may or may not encode functions needed for their own transposition. The second class consists of the larger transposons, some of which contain the simpler IS units at their termini and some encode transposition functions. Finally, Mu phage appears to represent the most complex Tn-like element. Mu can function like a transposon and can also exist extracellularly as a virus particle. The origins of transposons are unknown, but the similarity of the repeated recipient site sequences, discussed earlier, suggest the existence of three evolutionarily separate classes of transposable elements; namely, those with 5, 9, or 11 bp repeats. From available evidence, it appears that virtually any DNA segment that is flanked by identical IS units comprises a new transposon (71,89, M. Guyer and J.L. Rosner, personal communication). Since IS units are normal constituents of the bacterial chromosome and these units readily transpose, virtually any gene on the bacterial chromosome should exist as a transposable element at various times; for instance, the *his-gnd* genes that have recently been found to transpose. This, and the fact that plasmids can mediate the rapid dissemination of genetic information to many widely varied genera, lead to the conclusion that many chromosomal genes are constantly being interchanged among different bacterial species. This idea is also supported by the many variable biochemical traits commonly displayed by clinical bacterial isolates.

B. *Plasmids: Recombinational Assemblages of Transposable Units*

In cataloging the properties of many transposons detected on plasmids obtained from many different bacteria, the evolution of plasmids largely through a process of exchange of discrete transposable units is clearly discernable. For instance, knowing the location of several transposons on the R1 plasmid, Kopecko et al. (64,65) deduced that the R100, R6, and R1 plasmids, though obtained from different parts of the world, carry some of the same transposons (Figure 12). Moreover, because the shared transposons were residing at an identical location in each R plasmid these plasmids probably evolved from a common ancestral plasmid. In another example, members of the highly homologous Tn*A* group of transposons have been found on plasmids in a wide variety of bacterial genera. Furthermore, the genes for resistance to virtually all clinically useful antibacterial drugs have been found on transposons. Hence, transposons and plasmids have played a major role in the dissemination of drug resistance genes and other medically relevant determinants (e.g., enterotoxin or K88 antigen synthesis) among bacteria. Finally, in addition to transposons, many plasmids such as the R1 family (Figure 12) carry IS elements which allow for the chromosomal integration of plasmids (e.g., Hfr formation, see below) and for the amplification of genes located between two similar IS elements (82).

C. *The Chromosome: Constancy in the Face of Change*

Transposable DNA elements can promote the rearrangement, often at relatively high frequency, of portions of the bacterial chromosome. The transposable elements IS*1*-IS*5* and γ-δ exist in *E. coli* K-12 in multiple copies, and comprise approximately 1% of the total chromosome (Table 3). It is likely that these or similar transposable elements are largely responsible for the "spontaneous" chromosomal alterations in *E. coli* K-12 as well as other bacteria. The known locations of IS elements on the *E. coli* K-12 chromosome and the F plasmid are shown in Figures 13 and 14. The results of electron microscope studies of various F plasmid derivatives indicate that

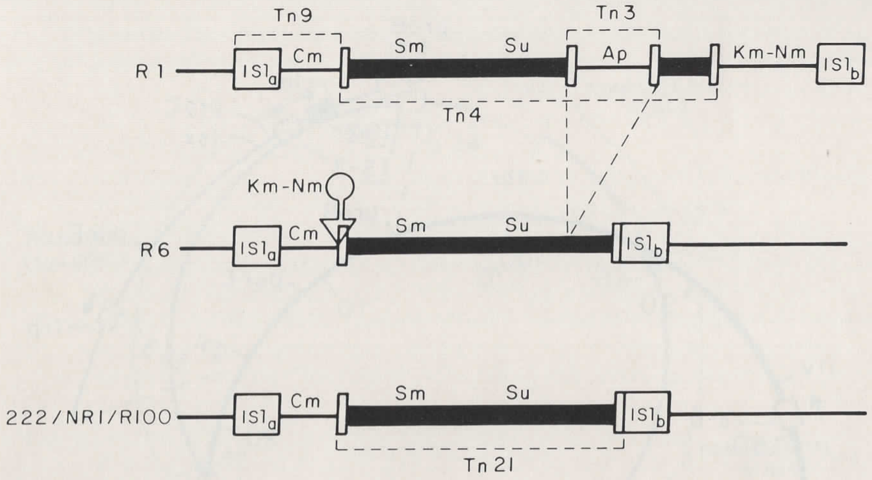

Figure 12. Relationship of r-determinant regions on R1, R6, and 222/NR1/R100 plasmids. Only the r-determinant region of these plasmids is depicted; direct repeats of IS1 were found to bracket the r-det *region (52,82). R1, R6 and 222/NR1/R100 are plasmids that were obtained from different bacteria isolated initially in England, Germany and Japan, respectively. The same transposable sequence encoding chloramphenicol resistance (Cm) is located in all three plasmids, as well as the larger transposon encoding streptomycin (Sm) and sulfonamide (Su) resistance. However, in the R1 plasmids the Tn3 (Ap) element is inserted within the Sm, Su transposon and now comprises the composite transposon Tn4. The R1 plasmid also carries an additional segment that encodes resistance to kanamycin-neomycin (Km-Nm) and which is deleted spontaneously at a high frequency, but has not been shown to transpose (63,64). Finally, the R6 plasmid carries an inserted segment of transposable genes encoding kanamycin/neomycin resistance located between the Cm and the Sm, Su transposons (see 64,65).*

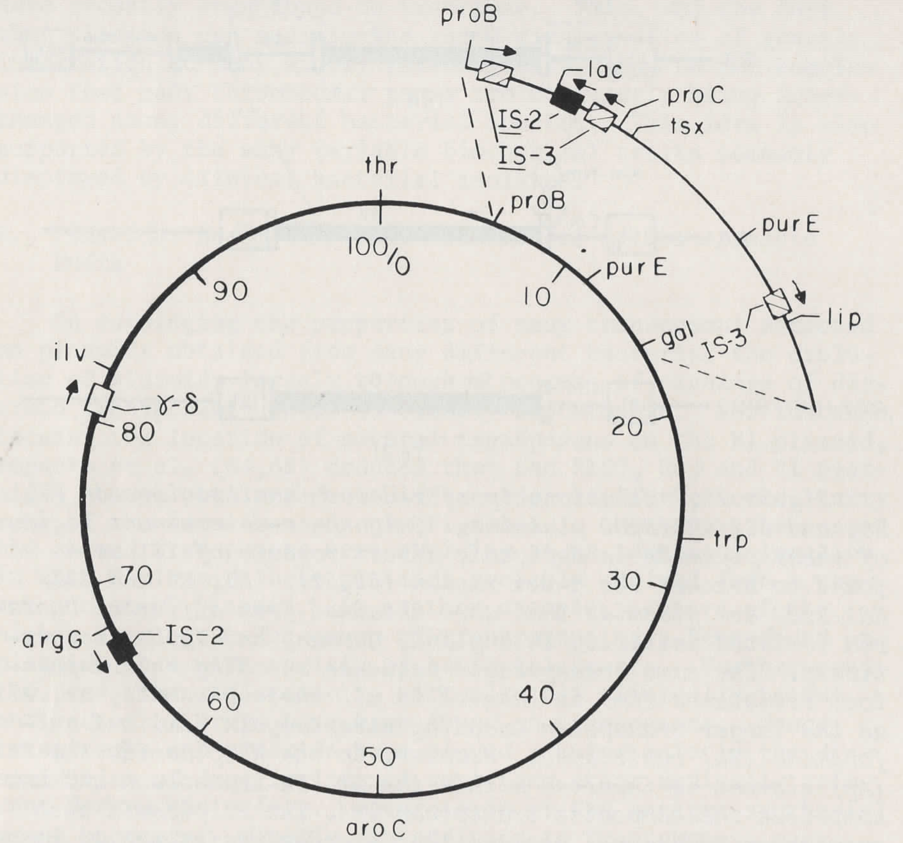
proB
lac
proC
tsx
IS-2
IS-3
thr
proB
purE
100/0
90
10
purE
lip
gal
IS-3
ilv
γ-δ
80
20
trp
70
30
argG
IS-2
60
40
50
aroC

Figure 13. Locations of insertion sequences acting as recombinational "hotspots" for Hfr formation. The circular linkage map of E. coli *K-12 is adapted from Bachman (4). N. Davidson and co-workers (27) deduced the identities and physical orientations of the insertion sequence elements that are actively involved in Hfr formation (indicated by shaded blocks and arrows in the figure) at six different locations on the* E. coli *genome. Although by DNA-DNA hybridization studies, the* E. coli *K-12 chromosome was estimated to carry five copies of IS2, the locations of only two such sequences are known. Twenty-seven different Hfr strains are thought to have been formed by F integration at one of the sequences mapped above. It is likely that other known Hfr strains were produced by F integration at other presently unmapped insertion sequences located on the chromosome.*

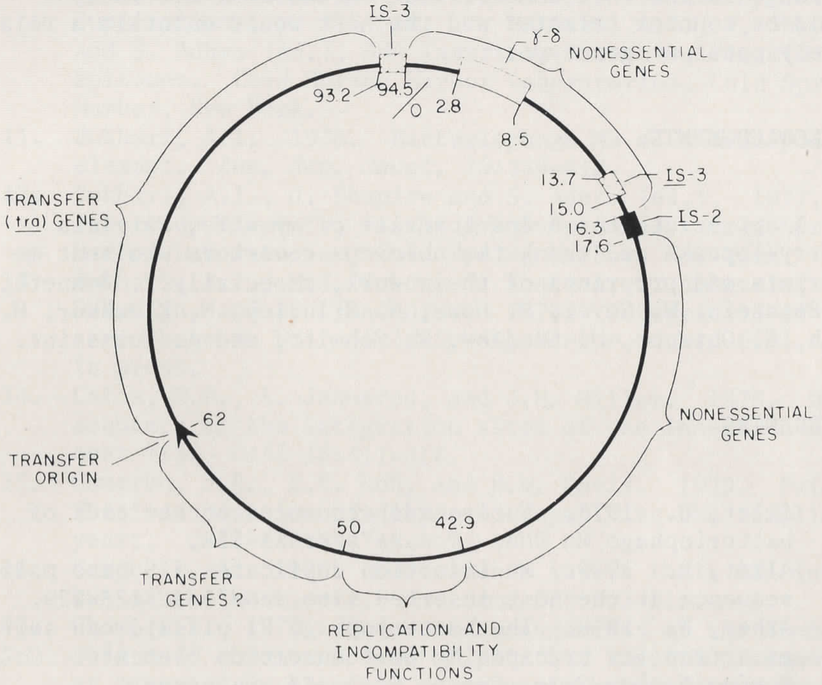
IS-3
γ-δ
NONESSENTIAL GENES
93.2
94.5
0
2.8
8.5
13.7
IS-3
15.0
16.3
IS-2
17.6
TRANSFER (tra) GENES
62
TRANSFER ORIGIN
NONESSENTIAL GENES
50
42.9
TRANSFER GENES?
REPLICATION AND INCOMPATIBILITY FUNCTIONS

Figure 14. Structural map of the F plasmid. Locations of IS sequences and other F functions are given in kilobase coordinates (27).

Hfr formation is the result of homologous pairing and recombination between IS elements on the F plasmid and identical IS units on the bacterial chromosome (27). Hfr polarity may be a direct consequence of the orientation of the homologous sequences on each parental molecule. These are further examples of the involvement of transposable elements in bacterial evolution. Without further justification, it appears obvious that chromosomal rearrangements occur quite often in bacteria. Why, in the face of such rapid macro-evolutionary genetic reshuffling, do bacteria appear to remain relatively constant in genetic makeup? One may speculate that over millions of years small chromosomal regions have been shuffled into their most stable relationships with neighboring DNA regions. Unless the environment changes and places new selective pressures on the host, most large rearrangements would cause instability of the chromosome and result in secondary, tertiary, and further alterations which would very probably be detrimental to the host. Thus, in the absence of a change in the environment, most chromosomal alterations would be counter selected and the host would maintain a relatively constant genotype.

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PROPERTIES OF DRUG RESISTANCE TRANSPOSONS, WITH PARTICULAR REFERENCE TO Tn10

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INTRODUCTION

Two features of antibiotic resistance determinants of medically important bacteria help to explain the rapid emergence and spread of resistance among different genera: (I) the determinants are often carried on plasmids thereby expanding the range of species in which the drug resistance can be inherited by facilitating transfer and establishment in the new hosts; (II) many resistance determinants on plasmids are translocatable to other replicons (Table 1).

Thus, *in vivo*, the association of drug resistance determinants with translocatable elements is particularly significant for the rapid development of important phenotypes by widely different bacteria. It is well known that different genera of Gram negative bacteria harbor particular classes of plasmid identifiable by incompatibility typing (7). Plasmids in different incompatibility groups usually share little nucleotide sequence homology (11), and members of a group are often limited in the hosts that they can enter or in which they can be stably inherited. A recombination mechanism which stimulated the translocation of resistance genes might enable these to survive in a new host by allowing their incorporation into the genome of a plasmid indigenous to that species. This notion is exemplified by the spread of ampicillin resistance

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Table 1. The Diversity of Transposons

Transposon	*Size*	*Markers*[a]	*Repeated Sequences*	*Reference*
Tn1	*4.8 kb*	*Ap*	*Short, inverted (40 bp)*	*14*
Tn2	*4.8 kb*	*Ap*	*Short, inverted (40 bp)*	*17*
Tn3	*4.8 kb*	*Ap*	*Short, inverted (40 bp)*	*31*
Tn4	*20.5 kb*	*ApSmSu*	*Short, inverted (40 bp)*	*31*
Tn402	*5 Mdal*	*Tp*	*None detected*	*49*
Tn5	*5.2 kb*	*Km*	*Long, inverted (1.4 kb)*	*3*
Tn501	*5.2 Mdal*	*Hg*	*Short, inverted*	*2*
Tn 551	*5.2 kb*	*Em*	*Short, inverted (70-100 bp)*	*39*
Tn554	*6 kb*	*EmSp*		*43*
Tn6	*4.1 kb*	*Km*	*Short*	*3*
Tn7	*8.5 Mdal*	*TpSm*	*Short, inverted*	*1*
Tn9	*2.5 kb*	*Cm*	*Long (IS1, 700 bp)*[b]	*10*
Tn903	*3.1 kb*	*Km*	*Long (1050 bp)*	*42*
Tn917	*5.1 kb*	*Em*	*Short, inverted (280 bp)*	*52*
Tn951	*16.6 kb*	*Lac*	*Short, inverted (100 bp)*	*5*
Tn10	*9.3 kb*	*Tc*	*Long, inverted (1:4 kb)*	*25*
Tn1681	*2.9 kb*	*Ent*	*Long, inverted (IS1, inverted)*[b]	*51*
Tn1721	*7.2 Mdal*	*Tc*		*47*

[a] *The abbreviations for the antibiotic resistance and other markers carried by transposons are: Ap (ampicillin resistance), Sm (streptomycin), Su (sulphonamides), Tp (trimethoprim), Km (kanamycin), Hg (mercuric salts), Em (erythromycin), Sp (spectinomycin), Cm (chloramphenicol), Tc (tetracycline), Lac (lactose fermentation), Ent (enterotoxin).*

[b] *Tn9 and Tn1681 are flanked by the insertion sequence IS1. In Tn9 the IS1 copies are in the direct order, while in Tn1681 they are inverted.*

conferred by the TEM β-lactamase determinant located on Transposon, Tn*10*, which was originally associated with IncFII R . This element is responsible for resistance to β-lactam antibiotics in many enteric bacteria, *Pseudomonas aeruginosa*, *Haemophilus influenzae* (33), and *Neisseria gonorrhoea* (44).

Also an element related to the tetracycline resistance transposon, Tn10, which was originally associated with IncFII R plasmids in enteric bacteria has recently emerged in clinical strains of *Haemophilus influenzae* (19).

Detailed physical analysis of R plasmids in incompatibility group FII has revealed that the differences in resistance traits can be attributed to the acquisition of drug resistance transposons (Figure 1) (30). The plasmids R1, R6, and R100 have extensive nucleotide sequence homology in the RTF region (which carries genes for conjugational transfer and plasmid replication) and in the drug-resistance determinant (r-det) region. Each plasmid has Su^r, Sm^r/Sp^r and Cm^r in common. R1 and R6 encode Tc^r located on Tn*10* in the RTF portion and Hg^r in the r-det. They differ in that R6 specifies Km^r located on Tn*903*. R1 is different from R100 and R6 because it does not have Tn*10*, and because it is Km^r (the genes are in a different place from the Km^r determinant of R6 and are probably not translocatable). In addition R1 specifies Ap^r carried by a Tn*A* element which is inserted in the Hg^r region of the plasmid. Genetic and biochemical experiments have revealed that R1 carries an inactive mercuric ion resistance (*mer*) operon (8).

DEFINITIVE PROPERTIES OF TRANSPOSONS

The following characteristics are common to most, if not all, translocatable elements (23): (I) Transposons are capable of serial translocation. Thus transposons can move from one replicon to another serially without changing their structure. (II) The replicons participating in translocation need not have any nucleotide sequences in common. (III) Translocation occurs at normal frequencies in hosts deficient in homologous recombination. (IV) Transposons have characteristic structures. Most have been shown to possess inverted repeat sequences at their termini which can form stem-loop structures in electron microscope heteroduplex experiments (Figure 2). Some elements have long inverted repeat sequences (e.g., Tn*5* and Tn*10*) while others have short inverted sequences (e.g., Tn*A* and IS1). This property is discussed in more detail later. (V) Transposons can insert at many sites in the recipient replicon. Most show some degree of clustering in preferred regions, at preferred sites or both.

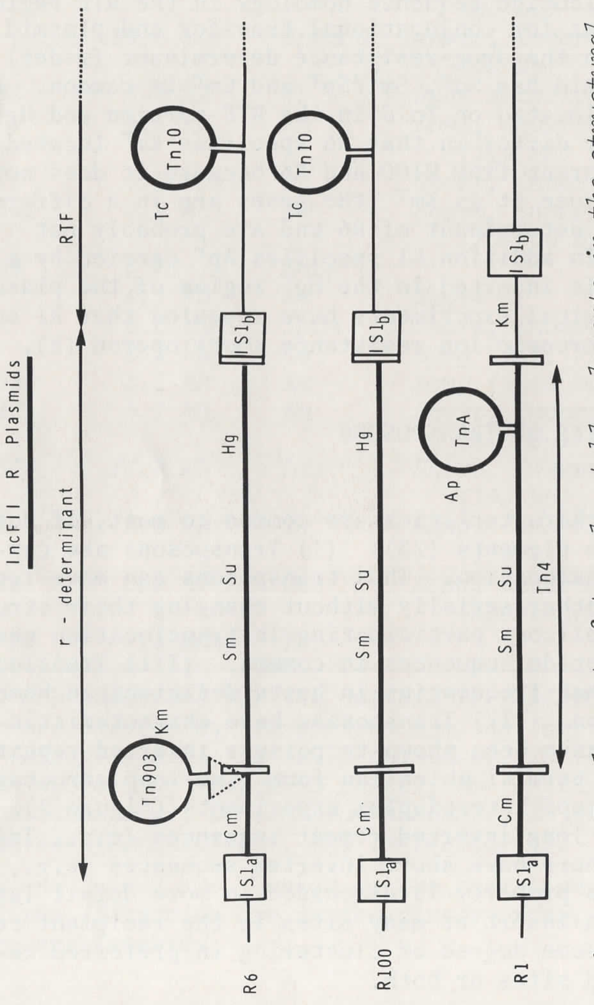

Figure 1. Involvement of translocatable elements in the structural evolution of R plasmids. The figure is redrawn from reference 30 to include recent data on the Hg^r *determinant (8). In addition to the transposons denoted, the entire r-determinant region of F-like R plasmids is translocatable (18). It is analogous to the Tn9 element in being flanked by directly repeated copies of IS1.*

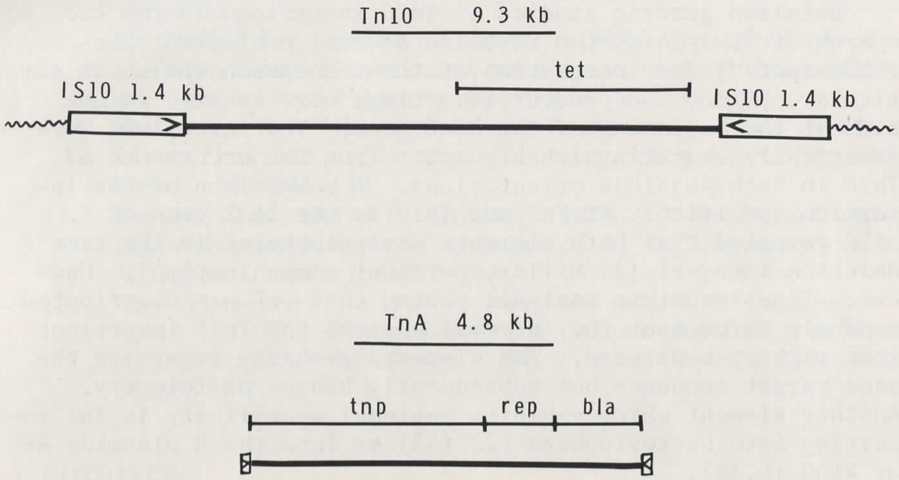

Figure 2. Structure of the drug resistance transposons Tn10 and TnA. Tn10 is a 9.3 kb element conferring tetracycline resistance (25). It is flanked by inverted repeats of a 1.4 kb sequence called IS10 (46). The approximate location of the tet *genes is indicated. TnA is the generic name of a family of transposons conferring ampicillin resistance. It is a 4.8 kb element with short, ca 40 bp inverted repeats. The locations of the TnA-encoded functions are shown. The abbreviations are as follows:* tet, *tetracycline resistance gene(s);* bla, *β-lactamase gene;* rep, *putative repressor of translocation;* tnp, trans-*acting transposition function.*

PROPERTIES OF TRANSPOSON-GENERATED INSERTION MUTATIONS

During translocation a transposon may insert into a structural gene in the recipient DNA molecule to cause a mutation. Thus Tn*10* causes auxotrophic mutations at a frequency of 1% when it translocates into the *E. coli* or *S. typhimurium* chromosomes (25,28). Insertion mutations are absolute, non-leaky lesions which are usually revertible to give a wild type phenotype with loss of the inserted transposon. The presence of a selectable phenotype facilitates genetic manipulations of the inactivated region (e.g., transduction of the mutation to a new host and promoting deletions in adjacent regions; 27) and the presence of the characteristic transposon structure allows physical mapping of the inactivated gene. This is proving to be particularly useful in the analysis of previously uncharacterized systems or in those in which mutations were difficult to isolate by conventional techniques (8,37).

Detailed genetic studies of Tn*10* insertions in the *his* operon of *S. typhimurium* revealed several preferred sites ("hot-spots") for integration of the transposon (28). In particular, 50/130 independent insertions were located in one site at the beginning of the *his*G gene. The insertions were genetically indistinguishable apart from the occurrence of Tn*10* in both possible orientations. A comparison of the insertion specificity of Tn*9* and Tn*10* in the *lac*Z gene of *E. coli* revealed that both elements were clustered in the same deletion interval (J. Miller, personal communication). However, fine-structure analysis showed that Tn*9* was distributed randomly throughout the interval whereas the Tn*10* insertions were tightly clustered. The elements probably recognize the same target sequence but subsequently behave differently. Another element which exhibits regional specificity is Tn*A* inserting into bacteriophage P22 (53) or into the R plasmids R6 or R100 (8,32).

THE DIVERSITY OF TRANSPOSONS

Many antibiotic resistance determinants of Gram negative bacteria are associated with translocatable elements (Table 1). Recently the drug resistance transposons Tn*551*, Tn*554* and Tn*917* have been described in Gram positive species. In addition, phenotypes other than antibiotic resistance can be specified by plasmid-borne transposons, for example, lactose fermentation (Tn*951*) and production of the heat stable enterotoxin of *E. coli* (Tn*1686*).

A prerequisite for translocation seems to be that the transposons be flanked by identical, but inverted sequences [also see Shapiro, this volume]. Thus mutants of Tn*A* and Tn*10* in which one repeat sequence has been deleted can no longer translocate (15). As mentioned previously, two classes of translocatable elements can be distinguished on the basis of the lengths of the terminally repeated sequences. Thus Tn*A* and IS1 have short sequences of 50 bp or less. The inverted repeats of Tn*A* were originally detected by electron microscope heteroduplex experiments (17), whereas the shorter (*ca* 30 bp) terminal repeats of IS1 were discovered by DNA sequencing (41).

There is an important difference between the short repeats which presumably have a purely structural role, possibly providing recognition signals for site-specific recombination enzymes, and the longer repeats of Tn*5*, Tn*9* and Tn*10*. In addition to the recognition role of their outside ends, the longer repeats are implicated in specifying functions involved in translocation (36,38).

The one apparent exception to the rule that translocatable elements must be flanked by inverted repeats may seem to be Tn*9* which is composed of direct repeats of the insertion sequence IS1 flanking the chloramphenicol resistance gene. However, IS1 itself is bounded by a 25 bp nearly perfect inverted repeat (41). This means that Tn*9* is in fact flanked by the short IS1 25 bp inverted repeats, and that this is true regardless of the orientation of the IS1 elements.

The r-det region of F-like plasmids is also bounded by directly repeated copies of IS1 (Figure 1). The entire r-det sequence has been shown to be transposable to bacteriophage P1 (18). Subsequently deletions removed part of the r-det to render the P1::r-det DNA more easily packageable. Tn*9* may have been formed by this process.

The longer inverted repeats of some transposons may be able to function as independent translocatable elements analogous to the IS sequences of *E. coli*. Indeed, Tn*9* and Tn*1681* are flanked by IS1 (35,51). However, the long inverted repeats of Tn*5* and Tn*10* do not correspond to any known IS sequence in *E. coli* (4,46). There is some evidence that the IS10 sequences of Tn*10* can behave as independent IS elements (26,50). Thus in a derivative of R6, R6-5, an additional copy of the IS10 element flanking Tn*10* has inserted into the *tet* genes to cause their reversible inactivation.

In conclusion, the transposons with the large (>700 bp) inverted repeats can be considered to be composite structures, comprising two insertion sequence-like elements which may be individually capable of translocation and which may encode specific functions involved in translocation, working in concert to transpose the intervening DNA sequences carrying the drug resistance genes.

DNA SEQUENCE ANALYSIS OF Tn*10* INSERTIONS IN BACTERIOPHAGE LAMBDA

In an effort to understand more about the molecular mechanism of Tn*10* translocation, six independent insertions in the *c*I gene of λ were examined (24). Junction fragements representing the joints between Tn*10* and the *c*I gene were obtained by elution from polyacrylamide gels following digestion with restriction enzymes. Relevant portions were sequenced by the method of Maxam and Gilbert. It was of particular interest to study target sequences at one site into which four of the six insertions had occurred. This region may be a "hot-spot" for Tn*10* insertion similar to the site detected genetically in the be particularly useful in the analysis of previously uncharacterized systems or in those in which mutations were difficult to isolate by conventional techniques (8,37).

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*Salmonella typhimurium his*G gene (28).

1. *Tn10 Generates a Nine Base-Pair Duplication of the Host Sequence at the Site of Insertion*

When Tn*10* integrates into a new DNA molecule it generates a direct duplication of a nine base-pair sequence at the insertion site. At the putative *c*I hot-spot the 9 bp sequence was the same in each of the four cases, showing that the insertion had occurred at precisely the same position. The duplicated sequence was different for the two other insertions. Most insertions of translocatable elements so far examined occur with either a 5 bp (Tn*3*, IS2; refs. 40,45) or a 9 bp duplication (Tn*9*, Tn*903*; refs. 20,42) of host target gene sequences; one 11 bp duplication has also been reported (13). This is seemingly a feature of the integration process during translocation and may be caused by a staggered cleavage of the target site and repair of the single stranded gaps as has been suggested in two molecular models for translocation (12,48).

The other possibility for the formation of the direct duplication of the host's sequences is a Campbell-type integration event with a circular transposon intermediate joined by a 9 bp sequence which recognizes a homologous 9 bp sequence in the recipient molecule. A reciprocal recombination event would result in the insertion of the transposon flanked by a directly duplicated 9 bp sequence. The available evidence suggests that the 9 bp duplication is not formed in this fashion: (i) the 9 bp sequences from different Tn*10* insertion sites in the *c*I recipient gene have no obvious similarity, even though the parental transposon was located at the same donor site; (ii) elements from different sites in a donor replicon (with presumably different 9 bp sequences) can recognize the same hot-spot in the recipient; (iii) a Tn*10* element, constructed genetically, which no longer carries flanking 9 bp repetitions of recipient DNA, is still capable of normal translocation (24).

2. *Analysis of Sequences at the Ends of Tn10*

The order of the first 80 bp of the outside ends of the IS10 element of Tn*10* are now known (24). The following features deserve comment. (I) The ends of Tn*10* are rich in A+T base pairs (67% A+T). (II) Translocation is an absolutely site-specific recombination event with respect to the translocating element. Each of five different insertions tested contain the same nucleotide pair of Tn*10* joined to the host target gene sequences. (III) Two 10 bp inverted repeat sequences were detected in the outermost 80 bp at the ends of Tn*10*. The role of these short repeats is not known. (IV) Some limited nucleotide sequence homology exists between the ends of Tn*10* and other elements that generate 9 bp direct duplications of the host's sequence during integration (IS1, Tn*903*; ref. 24).

FUNCTIONS ENCODED BY TRANSPOSONS

The notion that transposons specify functions involved in their own translocation was first proposed by Heffron, Bedinger, Champoux, and Falkow (15). Transposon *A* determines two proteins (apart from β-lactamase) that have been implicated in the translocation process (6,15,16). Lesions in the centre of Tn*A* occur in a putative regulatory gene (Figure 2) which specifies a 19000 dalton protein (6). This element is thought to be involved in controlling the expression of a gene whose product is required for translocation. Furthermore, some deletions in the central part of Tn*A* result in an aberrant translocation product during conjugational translocation assays involving translocation from a multicopy donor plasmid to a derivative of F factor (9). The Tn*A* donor replicon becomes fused into the mobilizing F plasmid and is flanked by directly repeated copies of the transposon. Some consider this structure to be an intermediate in translocation which cannot be resolved in the case of the Tn*A* mutants (48; F. Heffron, R. Gill and S. Falkow, personal communication).

TRANSLOCATION FUNCTIONS DETERMINED BY TRANSPOSON 10

Our recent experiments have concerned the molecular genetic analysis of functions encoded by the tetracycline resistance transposon Tn*10*. The two approaches taken in the generation *in vitro* of lesions within Tn*10* are depicted in Figure 3. In both cases the starting point was the wild-type Tn*10* element located at the *his*G hot-spot in a multicopy ampicillin resistance *his*OGD plasmid. Mutations were either generated in the intact transposon-carrying plasmid or in plasmids which carried the separated right and left "halves" of Tn*10* (Figure 3). Tn*10* elements comprising combinations of right and left half variants were then regenerated by cloning the *Eco*R1 fragments into the *Eco*R1 cloning vector λgt$_7$. Alternatively, complete plasmid-located Tn*10* derivatives were homogenotized onto a previously constructed λgt$_7$*his*OGD phage. The frequency of translocation of Tn*10* from the λgt$_7$*his*::Tn*10* phages into the

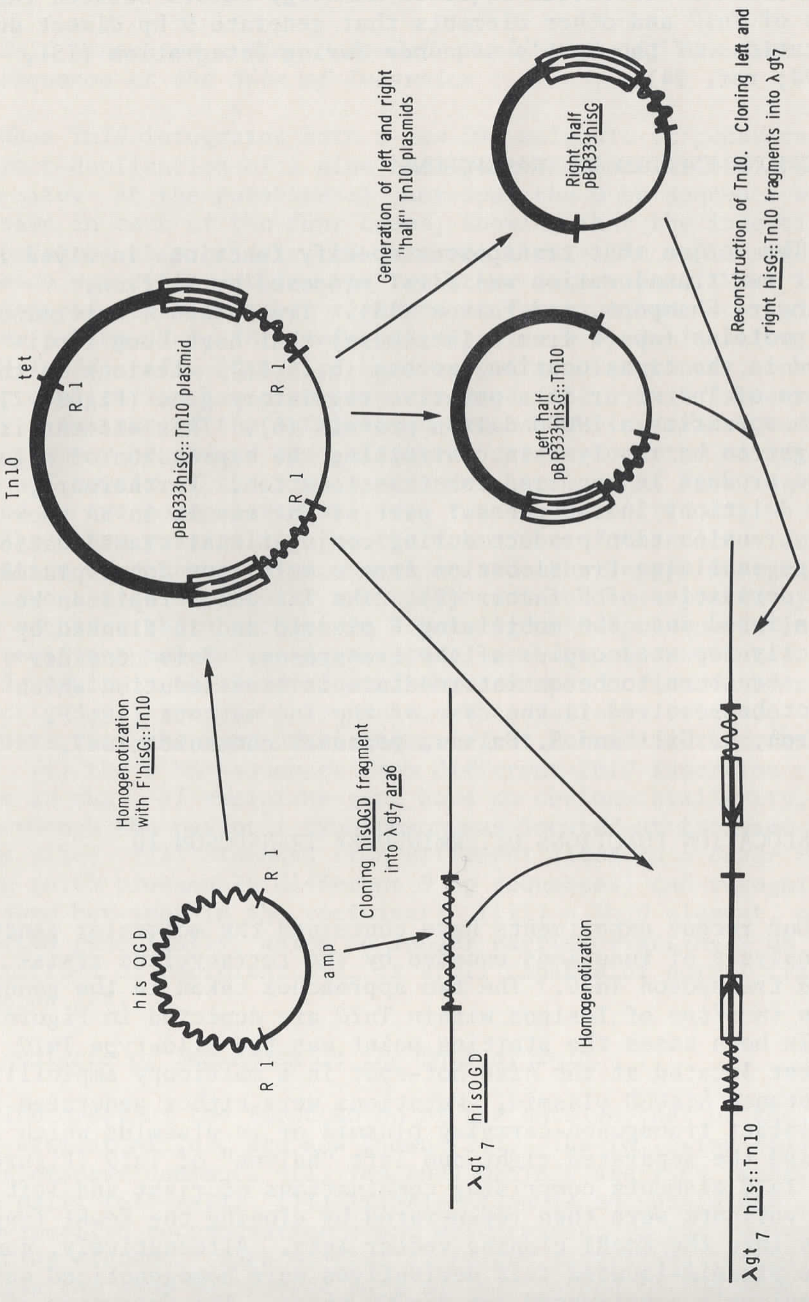
Tn10
tet
R1
R1
R1
R1
pBR333hisG::Tn10 plasmid
Homogenotization with F'hisG::Tn10
his OGD
R1
R1
amp
Cloning hisOGD fragment into λgt7 ara6
λgt7 hisOGD
Homogenotization
Generation of left and right "half" Tn10 plasmids
Left half pBR333hisG::Tn10
Right half pBR333hisG
Reconstruction of Tn10. Cloning left and right hisG::Tn10 fragments into λgt7
λgt7 his::Tn10

Figure 3

E. coli chromosome was then estimated (Table 2).

The location of deletions of Tn*10* generated *in vitro* are shown in Figure 4 and their effects on the frequency of *tet* translocation (Table 2) can be summarised as follows. (i) Deletions in the central (non-repeated) region of Tn*10* and deletions which extend to the *Acc*1 site in the left IS10 did not affect the frequency of translocation provided that the right IS10 was unchanged. (ii) A deletion which extended to the *Bc*I site located 65 bp from the outside end of the left IS10 (*del* 12) did not adversely effect translocation. (iii) Removal of the right IS10 resulted in an element which could no longer transpose, presumably because of a prerequisite for an important site at the very end of the element. (iv) Derivatives with the *Acc*1 site in the right IS10 filled in (*ins*1, putative frameshifts) were defective in translocation. Deletions in the right IS10 (*del* 10 and *del* 11) had a similar effect. When combined with deletions in the left IS10, the transposons were completely deficient in translocation. When the left IS10 was unaltered, the frequency of translocation was reduced by 50-fold.

Complementation tests, currently in progress, will directly demonstrate which of the lesions affect transposition functions and which affect important sites.

The simplest explanation for these results is that the right IS10 element encodes an essential transposition function. A similar function is expressed less efficiently by the left IS10. Recently it has been shown that the inverted repeats of Tn*5* are similarly heterogeneous in their capacity to encode Tn*5*-specific translocation functions (38; S. Rothstein and W. Reznikoff, personal communication).

Figure 3. Construction of gt_7hisG*::Tn*10*λphages. The diagram depicts the two routes employed in the construction of derivatives of* gt_7 *harboring variant Tn*10 *elements. Homogenotization of the Tn*10 *elements involves switching the transposon from one molecule to another using homologous recombination between* his *sequences located on either side of the transposon and corresponding sequences on the recipient molecule. The correct reconstruction of intact Tn*10 *elements from* Eco*R1 generated right and left Tn*10 *fragments was ensured by the regeneration of the* Tc^R *determinant. The wavy line denotes sequences containing the* his*OGD region of* Salmonella typhimurium. *The thicker lines correspond to Tn*10 *sequences. R1 =* Eco*R1 cleavage sites.*

Table 2. Translocation Frequencies Exhibited by Variant Tn10 Elements

Tn10 lesion[a]	*Frequency of translocation*[b]
del *1*	*4.1*
del *6*	*2.9*
del *2*	*2.5*
del *3*	*3.5*
del *4*	*1.3*
del *12*	*6.0*
ins *2*	*3.5*
del *8*	*0.01*
del *9*	*0.39*
del *10*	*0.10*
del *11*	*0.13*
ins *1*	*0.045*
wild type	*2.5*
del *2* ins *1*	*0.01*
del *3* ins *1*	*0.01*
del *4* ins *1*	*0.01*
del *4* del *10*	*0.01*
del *4* del *11*	*0.01*
del *6* ins *1*	*0.09*
del *7* ins *1*	*0.08*
del *7* del *10*	*0.03*

---

[a] *Each of the Tn*10 *variants described above with the exception of* del *12 was derived from a Tn*10 *element located at the major hot spot in the* hisG *gene (28). The* del *12 lesion was introduced into Tn*10 *located at the secondary* hisG *insertion site where the transposon was also in the opposite orientation. The wild type transposon at these sites translocated at the same frequency.*

[b] *The frequency of translocation of the variant Tn*10 *elements was estimated by infecting an* o^- *or a* p^- *amber mutant derivative of the* λgt_7hisG*::Tn*10 *(also* att^- *and* cI^-*) into an* Su^- *host and plating on agar containing tetracycline. Most of the resulting colonies were caused by translocation of the Tn*10 *from the lambda genome into the bacterial chromosome. The translocation frequency is expressed as the number of* Tet^r *colonies per input phage (x* 10^{-6}*).*

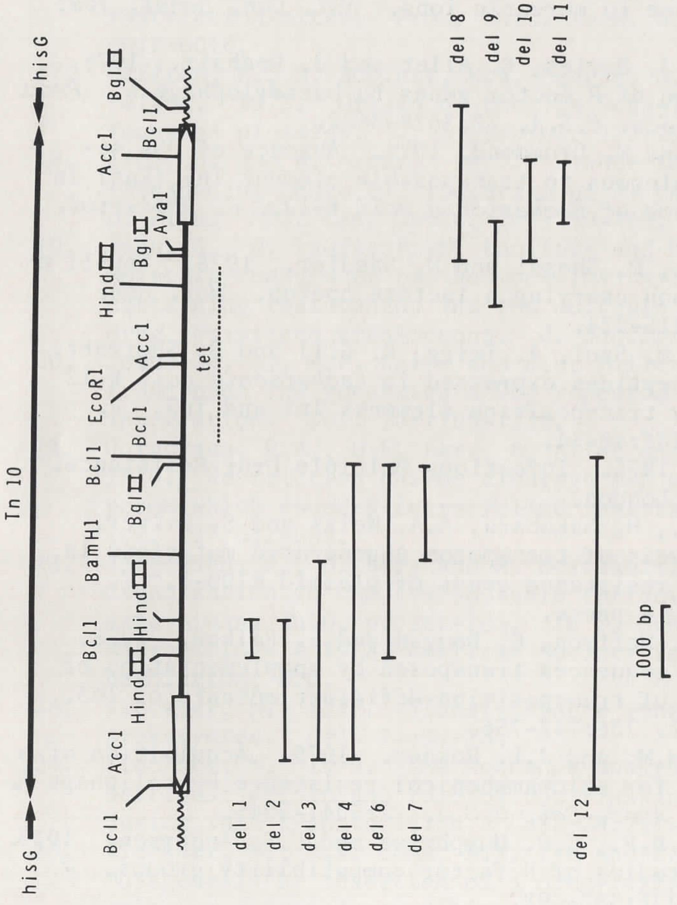

Figure 4. Location of in vitro *generated lesions of Tn*10. *The deletions* del *1-*del *8 and* del *10-*del *12 were generated by removing the DNA located between two restriction enzyme cleavage sites.* del *9 is a spontaneous deletion of ca 1100 bp which probably originates from the inside end of the right IS10. Mutations were also generated by filling in the* AccI *sites in the right and left IS 10 sequences to yield* ins1 *and* ins2 *respectively. If these lesions occur within the continuity of a gene, they will generate a frameshift mutation. The positions of the restriction endonuclease cleavage sites in Tn*10 *(with the exception of* BclI*) have been published (21,29). The tet genes are entirely contained between the* BglII *sites in Tn*10 *(21, unpublished data).*

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COMMENTS ON Tn*3*

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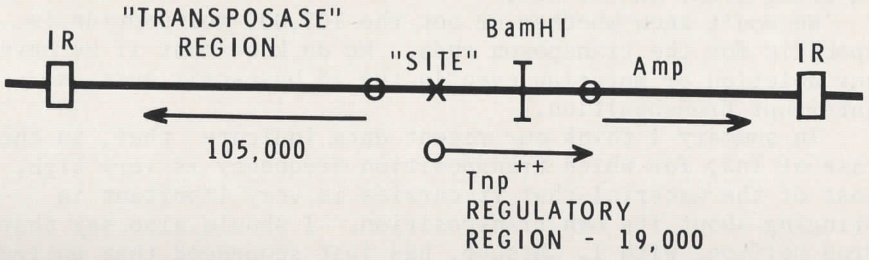
I would just like to expand on something which Tim Foster gave, which has to do with Tn*3*, and show some recent data obtained by Ron Gill in our laboratory. We've continued our collaboration with Fred Heffron since he left my group and we've come up with a little better picture of Tn*3*.

Tn*3* has got 38 base-pair inverted repeats at its ends. There is a *Bam* site which is useful since it bisects an important gene. The gene for *beta* lactamase (AMP) is stuck in a corner as if it's not really necessary, and it is transcribed in a rightward direction (diagram). The *Bam* site bisects a gene that encodes a 19,000 dalton protein. If you simply cut the molecule with *Bam* and remove the sticky ends and re-lygate, you get a strain which has a so-called Tnp^{++} phenotype. That is, it now transposes at a very high rate indeed - about 100 times normal. In addition, Fred Heffron has very nicely developed a deletion technique which has been a great boon to all of us. His technique permits the insertion of *Eco*RI sites at random within Tn*3*. So you not only could get a mutation in Tn*3*, but you could also map it quite precisely just by doing



Legend: ⟵ , *direction of transcription.*

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a simple enzyme digestion. He found that as he put EcoRI sites into the regulatory region (diagram) he could effect the production of a 19,000 dalton polypeptide and again it would lead to this particular Tnp^{++} phenotype. Adjacent to this regulatory region, and we don't know quite how long it is, there is something I will just call a "site". This "site" is important for the fidelity of transposition in the sense that if it is deleted, upon complementation, virtually all of the complemented transposons that you see show a "sandwich" effect, as described by Dr. Foster; where you have a whole Tn*A*, the replicon on which it was found (we usually use ColEI) and then another Tn*A* directly repeated. I just call that the "sandwich". Actually of the many mutations which do not affect the site giving the high frequency of transposition, about 10% are "sandwiches", all the rest are normal. The direction of transcription of the 19K protein is in the same direction as AMP.

The new thing that I would have to add is that we had a variety of mutations in a 'silent' region adjacent to the regulatory region and we were trying by genetic analysis to find complementation groups. We expected that there would be several functions that were important in transposition in this region since any kind of interruption in the DNA sequence would lead to a transposition-defective phenotype which, however, should be complemented in *trans*. However, we could not get any complementation whatsoever. To make a very long story short, it turns out that there is a promoter here and it simply encodes for one enormous polypeptide which is about 120 K daltons M.Wt., which we are going to call, for lack of a better word, a putative transposase. Any kind of interruption in this leads, as I say, to a transposition-deficient phenotype. If the 'transposase' gene is deleted then the 105,000 polypeptide that I've talked about is decreased in mass or truncated. So, it should now be possible to get the 105,000 polypeptide out, at least so that it's radiolabelled, and now do binding studies, and then see perhaps just what is its function. One would like to think, in a simple sense, that it may just be a kind of endonuclease, but it's not yet clear and one can't say anything about it for now.

We don't know whether or not the 105,000 polypeptide is specific for the transposon ends. We do know that if we have any deletion or mutation even in the 38 base-pair ends, we interrupt transposition.

In summary I think our recent data indicate that, in the case of Tn*3*, for which transposition frequency is very high, most of the material that it carries is very important in bringing about its own transposition. I should also say that Fred Heffron, with I. Ohtsubo, has just sequenced that entire Tn*3* from one end to the other. It's interesting that the sequencing data give precisely the same organization of Tn*3* exactly corresponding to what Ron Gill has deduced from genetic analysis and polypeptide synthesis in minicells. So I think there are two sets of complementary data that indicate that we're on the right track.

A MODEL FOR THE GENETIC ACTIVITY OF TRANSPOSABLE ELEMENTS INVOLVING DNA REPLICATION[1]

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INTRODUCTION

Earlier papers have described the basic phenomenology of transposition and chromosome rearrangements involving transposable elements (Foster, Kopecko, this volume). Formally, this phenomenology amounts to a series of non-homologous recombination events joining the ends of transposable elements to other DNA sequences. Early ideas about mechanisms for such recombination events were based on the bacteriophage λ integration and excision system (8,10,23). In particular, three features seemed to be extremely important in both cases: (i) the independence from host general recombination (*rec*) functions; (ii) site specificity for recombination events (*att* in λ, and transposable element termini); and (iii) the requirement for particular phage- or transposon-specified recombination enzymes (31,40,51). Two other observations bolstered this parallelism: the fact that both λ *attP* and transposon ends serve as hot spots for deletion formation (5,45,47) and sequence homology between the *attP* site and the ends of IS1 (33). In addition, it is possible to show that transposable

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elements can replace the *att-int* system as a mechanism for λ prophage insertion into the bacterial chromosome (36).

Notwithstanding these important parallels, more complete data on both λ integrative recombination and the nature of transposition indicate that the underlying molecular mechanisms are actually quite different. In this paper, I will summarize the evidence which suggests that the insertion process for transposable elements differs from that of λ in two respects. These are: (i) a requirement for base sequence homology at the recombination site in λ integrative recombination, and (ii) a requirement for DNA synthesis in transposition events. I will also describe a molecular model for transposition which explains many observations on transposon-related chromosome rearrangements and the replication of bacteriophage Mu (6).

THE λ SYSTEM

In integrative or excisive λ recombination, specific phage and host proteins act to produce reciprocal recombination products between two DNA molecules when each of them contains a substrate region called *att* (Figure 1; 23). The efficiency of this reaction and its requirement for the phage *xis* gene product depend on the various *att* sites involved in the cross (23,53), and recent sequence data on these sites indicate that a major determinant of *att* substrate activity is the degree of sequence homology in a central 15 base-pair "core" region (4, 33,34). In addition, genetic experiments indicate that there are heteroduplex regions formed in recombination intermediates (54) and that integrative recombination initiated at the *att* sites can lead to recombination in nearby intervals, presumably by branch migration of a Holliday structure (12). Thus, two independent lines of evidence point to a major role for base-pairing in the λ system.

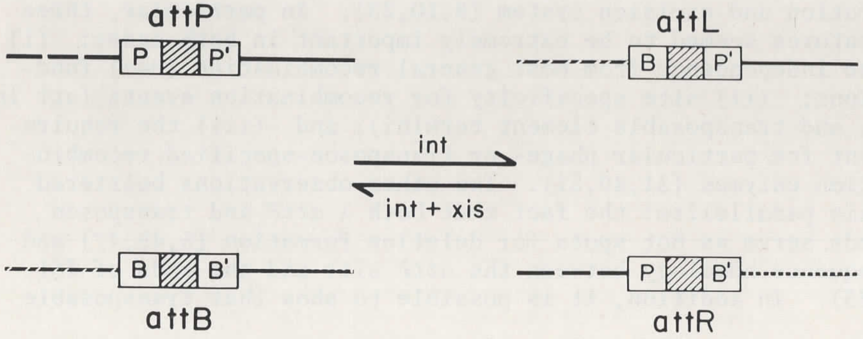

Figure 1. Integrative λ recombination. The att *sites normally involved in prophage λ insertion and excision are shown as boxes with a central 15 base-pair region of homology (cross-hatched). Note that the P,P',B and B' arms of the various* att *sites are not homologous (33) and that the reverse reactions have different requirements for the products of the viral* int *and* xis *genes (23).*

In vitro studies of λ integrative recombination show that the *attP* x *attB* reaction can proceed in the absence of nucleotide triphosphate hydrolysis and without gross loss of superhelical density if all substrates are supertwisted (38). This means that no DNA synthesis is necessary for the formation of the recombination products and that there is no free intermediate which contains either nicks or single-stranded regions. The absence of DNA synthesis in the recombination process is consistent with sequence analysis which shows that recombinant *att* sites are exact rearrangements of the parental *att* sites (4,33,34).

To sum up, the λ integration system appears to require base sequence homology and to involve no net synthesis of internucleotide bonds. Possible models to explain this process have been summarized by Enquist et al. (12).

THE ROLE OF DNA SYNTHESIS IN TRANSPOSITION

Recent data on the precise base sequences formed when a transposable element inserts into a new genetic site reveal a very different picture from the λ system. In all cases examined so far, the inserted element is flanked by duplicate copies of a short oligonucleotide sequence present at the target site *before* insertion (Figure 2). These duplications are either five base-pairs in length for IS2 (48), Tn3 (43), γδ (26) and Mu (1) or nine base-pairs in length for IS1 (9,25), Tn5 (2), Tn9 (30) and Tn10 (32). Studies of transposition of Tn9 and Tn10 from known sites indicate that the nine base-pair duplication does not reflect any homology between the oligonucleotides flanking the donor element and the target sequence (30,32). These results lead to two important conclusions: (i) transposition involves the synthesis of at least ten or eighteen internucleotide bonds (to generate the sequence duplication), and (ii) recombination between the transposable element and its target site does not involve the joining of homologous sequences. This second conclusion could also be

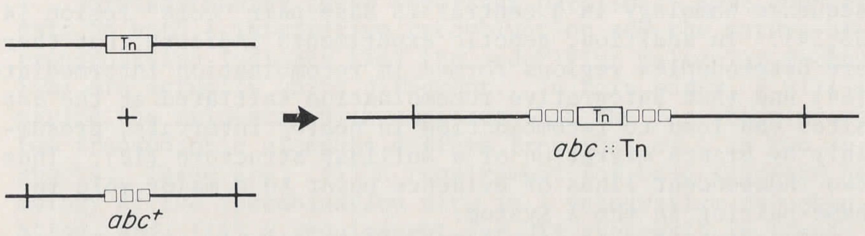

Figure 2. Insertion of a transposable element in a new genome location. The three boxes indicate the five or nine base pair sequence at the insertion site which is duplicated in the transposition process.

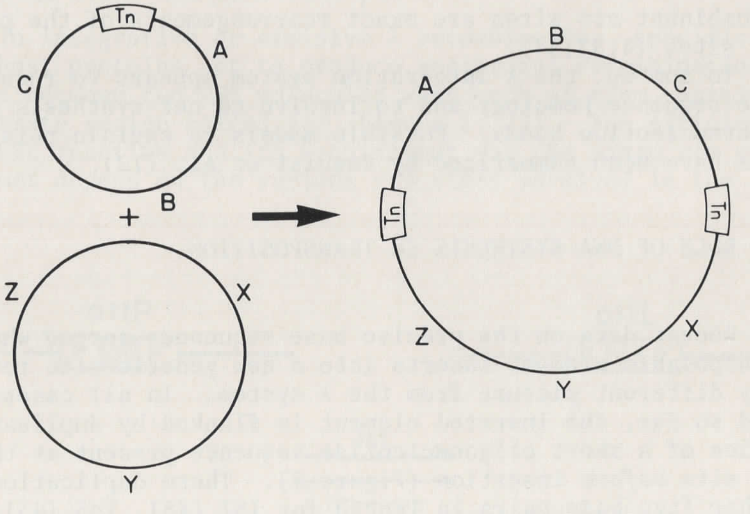

Figure 3. Replicon fusion.

drawn from the studies of Ohtsubo and Ohtsubo on the formation of deletions adjacent to the IS1 element (42).

The sequence data is not the only indication that DNA synthesis accompanies transposition or transposition-related recombination events. There are three other lines of evidence: (i) Transposable elements can serve to fuse two circular DNA molecules in a reaction called replicon fusion (Figure 3). In this reaction the two parental circles are joined in the cointegrate product by duplicate copies of the transposable element. Replicon fusion structures were first described in phage Mu-mediated integration of λ *gal* DNA into the bacterial chromosome (16,55) and have since been observed with certain mutants of Tn3 (21) and with IS1 either in λ::Tn9 phages (36, 52) or in plasmids (44). In the case of Mu and Tn3, we know that replicon fusion requires the same element-specified gene products as transposition (13,17,21,27). The appearance of duplicate elements suggests that the original element replicated in the replicon fusion reaction (17). (ii) Transposable elements are not lost from their donor site when they insert in a new target site. This genetic observation has been made in several laboratories working with different transposable elements. Bennett et al. showed that Tn3 transposition to new sites occurs at much higher frequency than its loss from the original site, which was virtually undetectable (3). Faelen and Toussaint recovered donor *Flac lacI::Mu* plasmids from cells where Mu had transposed to the bacterial chromosome (15). We have shown that *P. aeruginosa* cells which have received the Tn7 element inserted into the CAM-OCT plasmid also contain additional copies of the transposon at other sites in the genome while retaining Tn7 at its original location (18). So, the genetic evidence shows that transposable elements accumulate in the bacterial genome and thus apparently replicate before or during transposition. (iii) The replication of bacteriophage Mu is intimately associated with transposition. Genetic experiments show that a replicating Mu prophage inserts at new sites in the bacterial genome after derepression (46), and this process can be followed by physical methods which show that replicating Mu DNA rapidly becomes covalently linked to many different regions of the *E. coli* chromosome (35,49,56). Examination of viral DNA extracted from the progeny of a single Mu prophage shows that this process must have occurred, because virion molecules contain a random collection of host sequences attached to the Mu genome whereas the parental Mu genome was attached to a single site in the host chromosome (6,7,11). In addition, we know that Mu replication and Mu transposition involve the same Mu-specific gene product. Mutants with lesions in the Mu A gene are defective in the following events: insertion into the chromosome after infection (41), transposition from one prophage site to another (13,15), adjacent deletion formation (15), replicon fusion (17), and viral DNA replication after either infection or prophage derepression (55). Thus, both genetic and physical data indicate that transposition accompanies viral replication and that the phage transposition function is essential for replication to occur.

A MOLECULAR MODEL FOR TRANSPOSITION AND REPLICATION

The structural features of transposition events described by Foster elsewhere in this volume and the considerations outlined above lead to inclusion of the following elements in any mechanism for transposition: (i) replication of the transposable element and conservation or regeneration of the donor site; (ii) specific DNA cuts at the termini of the transposable element; (iii) staggered DNA cuts at the target site (as proposed by Grindley and Sherratt to explain the oligonucleotide duplication, 26); (iv) generation of replicon fusion structure as transposition intermediates or products of a process closely related to transposition.

Figure 4 presents a plausible model for transposition and replication which incorporates all of the features just listed. It has already been described in some detail (50), and the discussion below will emphasize a few points not clarified in the earlier presentation. As originally formulated, there are four steps which result in the transposition of the donor element (the heavy bars between markers A and B) to a target site between markers C and D (the squares indicate the oligonucleotide sequence destined for duplication): I. Cleavage of the donor molecule on opposite strands at the extremities of the transposable element and cleavage of the target molecule on opposite strands at the extremities of the duplicating oligonucleotide. These cleavages have opposite polarities so that one creates two 3' hydroxyl extensions and the other creates two 5' phosphate extensions. II. Ligation of the extensions so that each strand of the transposable element becomes continuous with a strand of the oligonucleotide target sequence. Note that this DNA joining reaction involves no base-pairing between donor and target molecules. The result of these ligations is the middle structure which is equivalent to a transposable element duplex flanked by two replication forks. This structure has only short single-stranded regions and should be stable in the absence of nucleolytic digestion.

Steps I and II probably occur in a concerted manner like other cleavage-ligation reactions (58) and are probably catalyzed by a protein aggregate; this would include an element-specific enzyme that recognizes its termini and a cellular enzyme that can make staggered five or nine base-pair cleavages when bound in the specific transposon-enzyme-target complex.

III. Filling of the short single-strand gaps and semiconservative replication of the transposable element duplex, followed by separation of complete daughter duplex molecules. This step results in the formation of two reciprocal recombination products (A-C and C-B) where the transposable element

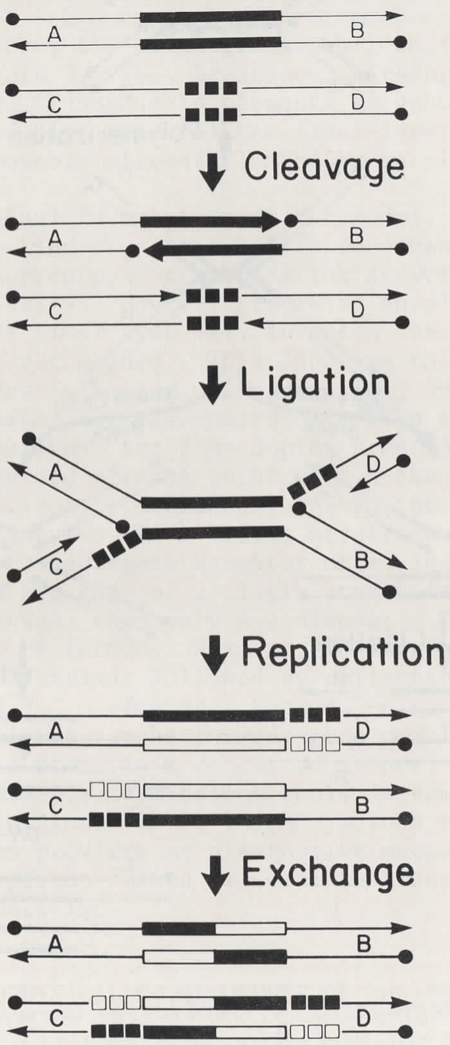

Figure 4. Transposition and replication. The various steps are explained in the text. The heavy bars represent the strands of the transposable element and the boxes indicate the target oligonucleotide sequence. Open bars and boxes represent DNA sequences synthesized in the transposition process. Arrowheads indicate 3' hydroxy termini and balls indicate 5' phosphate termini.

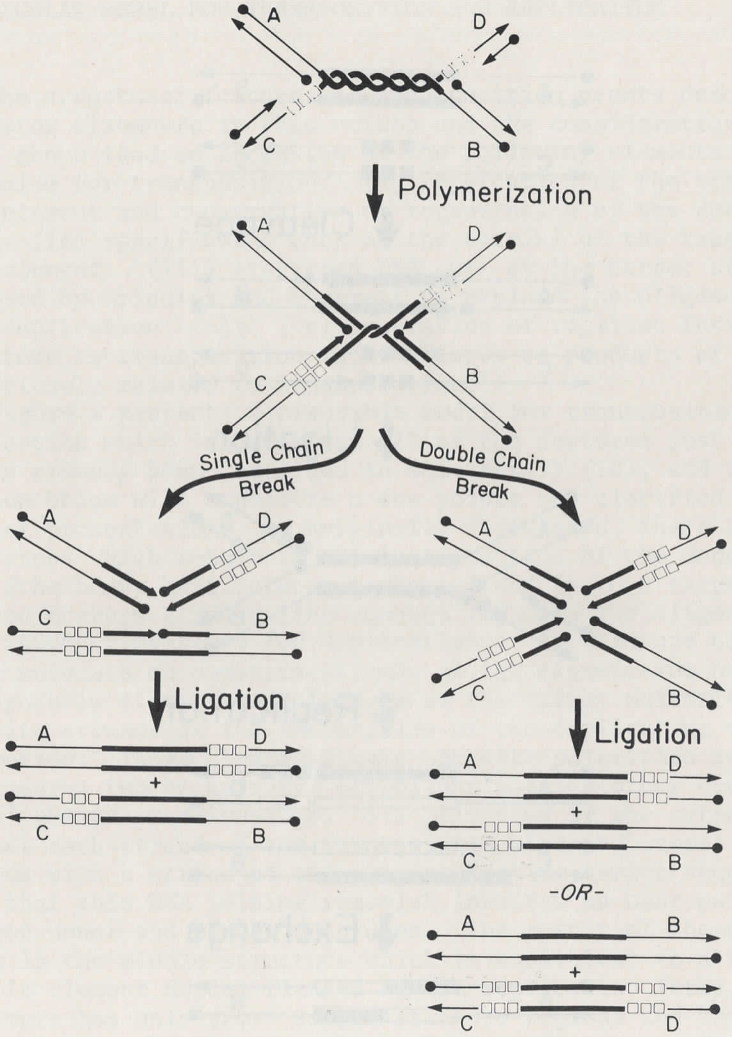

Figure 5. Segregation of replicated duplexes. The top figure shows the polymerization substrate as a double helix. Polymerization generates two gapped intertwined duplex molecules which must be cleaved to segregate progeny molecules. A breakage/ligation sequence in only one of the intertwined chains leads to two duplex molecules recombinant for the outside markers (left-hand branch). A breakage/ligation sequence in both intertwined chains leads to two duplex molecules which are either recombinant or *non-recombinant for the outside markers (right-hand branch).*

has served as the recombination "site." If donor and target molecules are circles, the recombinant products will form a single large circle of the following structure: A - transposable element - D - C - transposable element - B - A - etc. This is, of course, the cointegrate replicon fusion product depicted in Figure 3. IV. Reciprocal exchange between two newly-replicated transposable elements to generate (a) the original donor duplex and (b) the final insertion product with the transposable element flanked by an oligonucleotide duplication.

In the original formulation of the model, step IV was envisaged as some kind of site-specific recombination event to explain the occurrence of transposition as well as replicon fusion in Rec$^-$ cells. However, there is an alternative way of forming products after step III, in which the A - B and C - D markers are not recombined. This involves the segregation of daughter duplexes following polymerization, and is illustrated in Figure 5. After all base-pairs have been completed by the replication apparatus, the four duplex arms (A, B, C, D) will still be intertwined at some point within the transposable element or oligonucleotide sequences. As in the replication of all circular molecules *via* a Cairns structure, this tangle must be opened to segregate daughter molecules (20). If segregation involves breakage of a single strand followed by separation and religation, then only A - element - D + C - element - D duplexes will be formed. However, if segregation involves breakage of both strands followed by religation, then either A - element - B + C - element - D *or* A - element - D + C - element - B duplexes can be formed. Presumably, these reactions would also occur in a concerted manner, and the topoisomerases that catalyze termination could determine the exact geometry of rejoining. Thus, the enzymology of daughter duplex segregation provides an alternative mechanism for generating either replicon-fusion or complete transposition products from steps I-IV.

GENETIC AND BIOPHYSICAL CONSEQUENCES OF THE PROPOSED MECHANISM FOR TRANSPOSITION

The proposed transposition mechanism represents two new pathways of DNA metabolism in prokaryotic cells. First, it is a system for precise replication of a defined segment of linear, integrated DNA without an autonomous intermediate, and second it is a system for joining two completely non-homologous duplex regions using a transposable element as the recombination "site." It is not difficult to imagine that either of these pathways can confer selective advantages on specific organisms by providing means for chromosome rearrangements and selective DNA replication. There is already a fair body of evidence from work on bacteriophage Mu which supports this model for replication and recombination.

(i) Mu can generate chromosome rearrangements which include deletions and inversions as well as replicon fusions and transposition of random segments of the *E. coli* genome (13, 14). All of these rearrangements depend on the Mu A gene product and the presence of *both* ends of a Mu prophage (13,14, 15). Steps I-III of the model provide a simple explanation of replicon fusion (as outlined above) and also for adjacent deletions and inversions (Figure 6). In particular, the model for adjacent deletion formation in Figure 6 explains why this process should involve both ends of the transposable element and not just the end next to the deletion.

(ii) The model readily explains why Mu replication requires the A gene product if formation of the step II product is the only way to initiate specific Mu DNA replication. Moreover, the model explains two hitherto puzzling observations on the replication of Mu prophages following derepression: (a) the covalent bonds between Mu termini and the unique chromosome sequences adjacent to the prophage are not destroyed during replication (35), and (b) despite the maintenance of these linkages, Mu replication does not extend into the adjacent chromosomal sequences (57). An examination of the products of the step III replication will show that they fulfill both these requirements because *unreplicated* A and B duplex arms are both continuous with *replicated* element duplex.

UNSOLVED PROBLEMS AND A CONCLUSION

The model indicates at least two lines of future research on transposable elements in bacteria. The first is to show conclusively whether or not mechanisms based on replication are accurate representations of how transposition occurs. This will involve two approaches. An obvious one is to look for the formation of transposition products or intermediates *in vitro*, and initial experiments in this direction are underway in several laboratories. Another approach is to test the role of DNA replication functions on transposition *in vivo*. This will require methods for introducing transposition substrates and extracting products from cells under conditions where no DNA replication occurs. We are currently developing such methods in order to provide direct evidence that semiconservative replication functions are required for transposition.

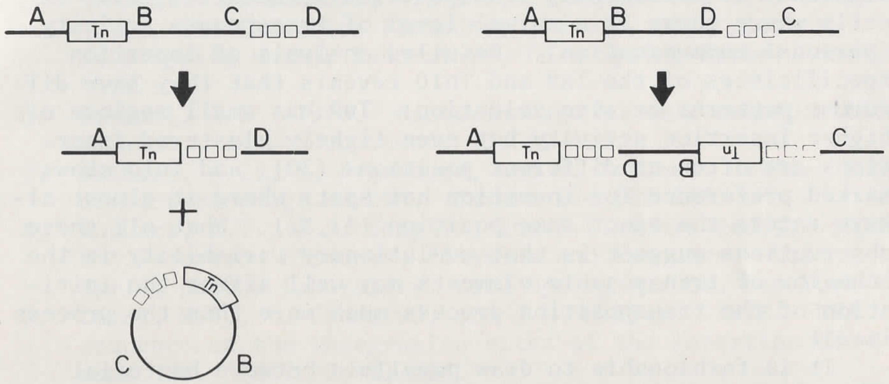

Figure 6. Consequences of cleavage, ligation and replication within a single molecule. The recombination events in the first three steps of Figure 4 are reproduced here without showing all the individual strands. (These are: A — Tn — B + C — oligo — D → A — Tn — oligo — D + C — oligo — Tn — B.) If A, B, C, D are arrayed as shown on the left, the products are a deletion from the transposable element (Tn) to the oligonucleotide target plus a circle composed of the deleted segment, the duplicated oligonucleotide and replicated transposable element. If A, B, C, D are arrayed as shown on the right, the product is an inversion of the material between the transposable element and the oligonucleotide target, with the inverted segment bracketed by opposed copies of the transposable element.

A second line of research centers on the formation of the initial donor-protein-target complex. There is a quantitative and a qualitative question which need answering: (i) What gene products and what features of substrate structure govern the rate with which complexes form? (ii) What features of the transposition process govern site selection for recombination events? There are very preliminary results on both these questions. The work of Heffron, Gill and their colleagues shows that at least one Tn3 gene product regulates the rate of transposition (21,27), apparently at least in part by transcriptional control of a transposase gene. Bacteriophage Mu appears to control the frequency of repeated transposition events by the product of the B gene because rearrangements involving two rounds of transposition occur at high frequencies with A^+B^+ phage but at low frequencies (characteristic of two independent events) with A^+B^- phage (15). Preliminary results of D. Berg and R. Laux with internal substitutions of Tn5 indicate that transposon sequences other than the termini are important in determining transposition frequencies, even in cells where there is a normal level of transposase activity (personal communication). Detailed analysis of insertion specificities of the Tn9 and Tn10 reveals that they have different patterns of site selection: Tn9 has small regions of higher insertion activity but even tightly clustered insertions are often at different positions (30), and Tn10 shows marked preference for insertion hot spots where it almost always enters the exact same position (31,32). What all these observations suggest is that evolutionary variability in the behavior of transposable elements may well affect the initiation of the transposition process much more than the process itself.

It is fashionable to draw parallels between bacterial transposable elements and similar genetic systems in eukaryotes, such as yeast mating type interconversions (28), *Drosophila* mutators and mitotic recombination elements (24), and *maize* controlling elements (37). Such parallels can be instructive, but they can also be misleading if followed too rigidly. I think that studies in bacteria on homologous recombination (22,29), "illegitimate" recombination involved in the formation of deletions, specialized transducing phage and tandem duplications (19,59), integrative lambdoid phage recombination (23,38), restriction enzymes (39) and transposable elements all point to an important conclusion -- namely, there are multiple pathways for both homologous and non-homologous recombination events, and living cells will make use of many of them.

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DISCUSSION

Novick: I am strongly committed to the idea that transposition is conservative, that is, the donor site retains a copy. However, it should be pointed out that there really isn't any clear evidence for it, because the alternative is that the donor replicon is destroyed and that you look at a second copy that is present in the cell at the same time. It's very difficult to rule that out. It's perhaps just worth mentioning as one of the problems still remaining in the system.

SHAPIRO: Sure.

Johnston: Does the model predict that a transposon will always be bordered by inverted oligonucleotides?

SHAPIRO: By *direct* repeats of very short oligonucleotides in the target DNA. At least in two cases now when Mu is inserted: one in the *lac* operon, and one in lambda, there are 5 base-pairs initially present in the target DNA which are subsequently duplicated on either side of the inserted Mu prophage. So in that way Mu seems to be similar to the other transposable elements.

Novick: Why would you suppose duplication is obligatory? That is, it's conceivable that you could imagine a blunt ended insertion rather than a staggered insertion.

Kopecko: That's the way the enzyme works.

Novick: Yes, but there doesn't seem to be any theoretical reason for that.

SHAPIRO: Alright; however, there are such enzymes in cells. It's known that DNA gyrase makes staggered cuts in DNA and that it exists covalently bound to the DNA as an intermediate while it's twisting up the DNA.

Kopecko: Mark Guyer has recent data on Tn9-promoted λ insertion onto an F plasmid, and he found that instead of a true cointegrate-type structure with a repeat of Tn9, it's just an IS1 repeat with one chloramphenicol resistance sequence at one end. So that's really not a true cointegrate except in terms of IS1. Also, the only time this event has been observed in small plasmids is on multi-copy ColE1 plasmids, and I wonder if their rapid replication has something to do with the formation of cointegrates.

SHAPIRO: Well cointegrates have now been observed with Mu integrating a non-replicating λ-*gal* circle into the chromosome. That was first seen, I think, over ten years ago now. Martha Howe recently cloned the ends of Mu in lambda and found that whenever she made a mutation with that lambda phage, Mu did not hop out of the lambda into the chromosome but formed what looked like the cointegrate of lambda, and so those are very big molecules. We've seen it, just as Mark Guyer has, in lambda with Tn9, at least the IS1 is duplicated in that process.

Falkow: You can take a mutant Tn3 and put it into something like F . . .

Kopecko: Put it on a big plasmid?

Falkow: Yes, it does the same thing.

SHAPIRO: Many Hfr's are probably formed by homologous recombination into the chromosome, but some Hfr's could be exactly this kind of event.

Willetts: It seems to me that one major problem with your model is: how does a particular transposition system recognise nicks that have been made, as you have suggested, by a cellular enzyme. That is, how does it know which of the 5-, 9- or 11-base pair targets is which unless it is itself making those nicks? Also, apparently no such enzyme has been described, as far as I know, and the nicks that are made are not really like restriction endonucleases because they don't have the symmetry which is associated with restriction endonucleases.

SHAPIRO: Well gyrase makes a 4 base-pair staggered cut and has some very broad sequence specificity, but I don't think it requires palindromic sequences such as restriction enzymes do. Anyway, it makes a lot of cuts in ColE1. I think transposable elements have evolved in various host strains and the postulated enzymes which make these staggered cuts are probably some kind of DNA topoisomerases which are active in various aspects of DNA metabolism. Perhaps it's in replication, say, and the elements have evolved in such a way that their specific nucleases probably interact with the specific nucleases of the cell. It's no good for the cell to have an enzyme which is always making 5 or 9 base-pair staggered cuts because the chromosome would fall apart. However, it could be that when that protein is in contact with a protein coded by one of these elements, a complex is formed and then the cuts are generated. It's known that in DNA gyrase the two subunits of the enzyme have to come together in order for them to have any activity on DNA at all. So protein-protein interactions can put these nucleases in the active configuration. That's why the short staggered cuts probably are not made all the time in the cell.

Willetts: But in fact all you're saying is that although there is only one protein involved, according to what Stan was saying earlier, it simply has two functions. Whether one function is to recognise the endonuclease that puts in the staggered cuts or it puts in those cuts itself, it has to be bifunctional.

SHAPIRO: Yes, but proteins have long been known to recognise other proteins which affect their catalytic properties.

Willetts: But it seems to be the weakest part of the model; there isn't any good evidence for that particular facet.

SHAPIRO: Except that those sequences do get duplicated.

Unidentified: Gyrase involves nucleotide hydrolysis, but your model doesn't involve hydrolysis?

SHAPIRO: No, λ integration doesn't require any nucleotide hydrolysis. If the model is in any way correct, obviously a lot of deoxyribonucleotide triphosphates must be hydrolysed in order to get a transposition product. I think that one of the predictions of the model is that it depends on semi-conservative DNA replication which is actually a very difficult thing to test. If you do any kind of assay where you ask for a biologically functional molecule to come out of a cell, you can't kill that cell. You have to go to special things like extracting DNA molecules and looking at what happens to those when they're present in the cell under totally non-permissive conditions.

Novick: Has anybody looked at *polA*-defective mutants for transposition?

SHAPIRO: Yes, I think they're okay.

Falkow: I'm interested in the situation where the presence of a transposon in a particular plasmid prevents the acquisition of a second copy of that transposon.

SHAPIRO: No, I don't think that's true.

Falkow: Well, it's certainly true for a plasmid; I don't know about the chromosome.

SHAPIRO: It's true for Tn*3* in small plasmids. Apparently two inverted repeats of the *beta* lactamase transposon can occur in plasmid R6K, but direct repeats don't occur and apparently they can't even be constructed. This may be because a site in Tn*3* is possibly involved in the termination of replication. So a single copy in a plasmid would be alright, but two copies may prevent the completion of plasmid replication. Obviously this may be wrong and it certainly is not by any means a complete description of everything that happens.

TRANSPOSABLE MERCURY RESISTANCE IN *Pseudomonas putida*[1]

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We have previously demonstrated that a plasmid, MER, specifying mercury resistance, occurs as part of a degradative plasmid aggregate in *Pseudomonas putida* (4). Here, we report the occurrence of the *P. putida mer* genetic region as a discrete element that can transpose from the chromosome of *P. putida* to plasmids including broad-host range antibiotic resistance plasmids, and from there, to the *Escherichia coli* chromosome and back to other plasmids. Such transposition events are independent of host recombination functions, and transposition of *mer* (designated Tn*1861*) on the chromosome of either *P. putida* or *E. coli* cells causes gene inactivation at or near the site of insertion. Bacterial strains used in this study, with their relevant genotypes, are listed in Table 1.

CHROMOSOMAL INTEGRATION AND MUTAGENIC POTENTIAL OF THE *mer* GENE

Resistance to mercuric ions is primarily mediated by a mercuric reductase that is specified by a Mer plasmid and converts inorganic mercury to metallic mercury which can be volatilized from the culture (7). Recent work of T.G.

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TABLE 1. Bacterial Strains Used

Strain		Plasmid	Genotype	Reference or Derivation
P. putida	AC77	MER	met ade mer[a]	4
	AC1000		met ade mer	this paper
	AC1001	RP4	met ade mer	this paper
	AC1006		met mer	Ade$^+$ spontaneous revertant
	AC1008		met thr ade mer	(Ade$^+$ revertant on casamino acid plates)
	AC1012	RSF1010	met ade mer	this paper
	AC574		trpF316 leu501	this paper
	AC1020	RP4::*Tn*1861	trpF316 leu501 mer	this paper
E. coli	AC548		thr leu thi trp recA	6
	AC80		thr leu met res mod	6
	AC1016		thr leu thi trp recA met mer	this paper
	AC1018	RSF1010	thr leu thi trp recA met pro mer	this paper
	AC1019	RSF1010::*Tn*1861	thr leu met res mod mer	this paper

[a] *The genotype* mer *symbol indicates that the strain was resistant to mercury but had no detectable plasmid DNA; the* mer *was assumed to be chromosomal.*

Kinschert and S. Silver (personal communication) demonstrated that a crude enzyme preparation of the MER-specified mercuric reductase from *P. putida* strain AC77 is immunologically similar to a large number of mercuric reductases specified by other plasmids such as the *E. coli* plasmid R100; the plasmid in *P. fluorescens* B69; and the transposon Tn*501* (1,14). Since we were interested in determining the molecular size and the nature of the genes in the MER plasmid, we attempted to isolate plasmid DNA from single colonies of strain AC77 that had been obtained by continuous long-term subculturing on nutrient agar containing a high concentration of mercury (160 µM). Repeated attempts using a number of different methods for the isolation of plasmid DNA (5,8,10,13) from strain AC77 failed to yield any detectable plasmid DNA from mercury resistant cells. Additionally, while none of the approximately 10,000 single colonies tested showed any detectable loss of mercury resistance, additional mutations causing phenotypes such as Ade$^-$, Trp$^-$, and Thr$^-$ were recovered at a frequency of about 10^{-2} to 10^{-3} per cell per generation in strain AC77.

To examine the genetic basis of stable resistance to mercury and the nature of the auxotrophy induced, one Ade$^-$ mutant of strain AC77 (AC1000, *met*$^-$, *ade*$^-$) was studied further. We found that (i) no plasmid DNA could be isolated from single colonies resistant to 225 µM Hg^{2+}; (ii) the mercury resistance determinant in strain AC1000 could not be transferred to other *P. putida* or *E. coli* cells at any detectable frequency ($<10^{-9}$/cell); (iii) strain AC1000 appeared to acquire additional leaky mutations at a frequency of about 10^{-3} per cell per generation (Table 2). The rationale for examining requirements of Ade$^+$ revertants of strain AC1000 is that if the

TABLE 2. Genetic Instability of Strain AC1000

Strain	*Selection media*	*Reversion frequency*	*Phenotype of Ade$^+$ revertants*
AC1000	*Minimal glucose + methionine*	10^{-8}	*Met$^-$*
	Minimal glucose + casamino acids	2×10^{-8}	*Met$^-$Thr$^-$[a]* *Met$^-$Thr$^-$Leu$^-$*

[a] *In general, Thr$^-$ or Leu$^-$ mutations were leaky; such mutants grew well with methionine and adenine, or methionine and threonine, but not methionine alone. Continued subculturing of such cells on L agar produced mutants that no longer grew on minimal glucose agar supplemented with casamino acids.*

Ade$^-$ mutation is caused by stable integration of the *mer* region on the chromosome at or near the *ade* locus, then it might be possible to isolate Ade$^+$ revertants in which the total *mer* region had been excised from the *ade* locus. However, this presupposed the occurrence of precise excision, as with Tn*5* (Km^R) and Tn*10* (Tc^R), to restore the original gene activity (2,3). The spontaneous reversion frequency of strain AC1000 to Ade$^+$ was about 10^{-8}/cell, and all Ade$^+$ revertants were fully resistant to mercury. Thus, mutagenic activity induced by the putative Tn*mer*, designated Tn*1861*, differed from that of Tn*5* or Tn*10* where reversion of auxotrophic mutations, originally caused by the insertion of the respective transposons, usually follows from precise excision of the transposons, and therefore, results in a concomitant loss of resistance to Km or Tc respectively.

Another difference between Tn*1861* and transposons known to induce mutations on the chromosome was the continued genetic instability of the Ade$^+$ revertants harboring Tn*1861*. Thus, when about 10^9 cells of strain AC1000 were plated on glucose minimal plates containing casamino acids and incubated for five to six days at 32^{o}C, about 6 to 8 colonies appeared in three days, all of which were Ade$^+$Met$^-$Hg^r and grew well on minimal glucose agar with methionine. Upon prolonged incubation, about 10 to 12 small colonies appeared that could grow on glucose minimal agar supplemented with casamino acids but not on glucose minimal agar with methionine (Table 2). We tested a few single colony isolates of such slow-growing revertants which were leaky and could grow on minimal glucose plates containing either methionine and threonine, or methionine and adenine, but not on methionine alone. Growth was much better with adenine than with threonine. Such Met$^-$Thr$^-$(Ade$^-$) cells continually produced other leaky mutants with additional requirements for other amino acids and vitamins. Therefore, Tn*1861* apparently occupied a primary site on the chromosome at or near the *ade* locus, but presumably could also occur with more than one copy on the chromosome. Ade$^+$ revertants possibly arose by precise excision from this primary site and possible insertion at a secondary site. On continued growth, another Tn*1861* copy could have transposed from a secondary site back to the primary site leaving a copy at the secondary site. Thus, the *thr* locus might be a secondary site from which the ready transposition of Tn*1861* onto the primary site (*ade*) produced cells with two leaky mutations allowing growth on either threonine or adenine supplemented media, but no growth when neither was present. These observations bear striking similarities to the proline-leucine instability in *Salmonella* reported by Dawson and Smith-Keary (9) on which their controlling episome hypothesis was based about a decade before transposons were first described.

TRANSPOSITION OF Tn*1861* FROM THE *P. putida* AC1000 CHROMOSOME ONTO OTHER PLASMIDS

To determine whether the mercury resistance character could be transposed from the chromosomal *ade::*Tn*1861* locus to various plasmids, including broad host range plasmids, and thereby be transmissible to members of other bacterial genera such as *E. coli*, two plasmids, RP4 and RSF1010 (12) isolated from *P. putida*, were introduced into strain AC1000 by transformation. The RP4 and RSF1010 plasmid DNAs were then isolated from the respective transformants (strains AC1001 and AC1012) and used to transform *E. coli* and *P. putida* recipients (Table 3). With selection for Cb^r among *E coli* recipients of RP4, about 20% also received the *mer* character. Similarly, in transformation of *P. putida* with RP4 DNA a significant proportion of the Cb^r transformants (15%) also acquired the mercury resistance character. When DNA (which would be chromsomal since no plasmid band was seen in extracts of strain AC1000) was isolated from strain AC1000 and used to transform *E. coli* or *P. putida* with selection for mercury resistance, no mercury resistant colonies were obtained.

The acquisition of mercury resistance by *E coli* and *P. putida* transformants receiving RSF1010 was less frequent, presumably because of the instability of Tn*1861* on RSF1010. Thus, when a single colony isolate of a Sm^{r}Hg^r *E. coli* transformant was grown in the absence of Hg^{2+} for about 20 generations and tested for the retention of Tn*1861*, about 50-70% of the Sm^r colonies appeared to have lost mercury resistance. Similar loss was detected in *P. putida* transformants. The extent of loss of Tn*1861* from RP4 was much less frequent, usually around 1-2% of the Cb^r colonies.

TABLE 3. Transformation of P. putida *and* E. coli *with RP4 and RSF1010 DNA Isolated from Strains AC1001 and AC1012*

Plasmid DNA	*Recipient*	*Selected marker*	*Transformant phenotype*
RP4	*AC80*	*Cb*^{r}	*Thr*$^-$*Leu-Met*$^-$*Cb*^{r}*Km*^{r}*Tc*^{r}*Hg*^{s} *(80%)*
			Thr$^-$*Leu*$^-$*Met*$^-$*Cb*^{r}*Km*^{r}*Tc*^{r}*Hg*^{r} *(20%)*
	AC574	*Cb*^{r}	*Trp*$^-$*Leu*$^-$*Cb*^{r}*Km*^{r}*Tc*^{r}*Hg*^{s} *(85%)*
			Trp$^-$*Leu*$^-$*Cb*^{r}*Km*^{r}*Tc*^{r}*Hg*^{r} *(15%)*
RSF1010	*AC80*	*Sm*^{r}	*Thr*$^-$*Leu*$^-$*Met*$^-$*Sm*^{r}*Hg*^{s} *(98%)*
			Thr$^-$*Leu*$^-$*Met*$^-$*Sm*^{r}*Hg*^{r} *(2%)*
	AC574	*Sm*^{r}	*Trp*$^-$*Leu*$^-$*Sm*^{r}*Hg*^{s} *(95%)*
			Trp$^-$*Leu*$^-$*Sm*^{r}*Hg*^{r} *(5%)*

GENETIC INSTABILITY IN *E. coli* DUE TO CHROMOSOMAL INTEGRATION OF Tn*1861*

To determine whether the presence of Tn*1861* in RP4 might cause genetic instability in *E. coli*, a single colony isolate of a transformant of strain AC548 (*recA*) carrying RP4::Tn*1861* was grown overnight, and single colonies were examined for induction of additional auxotrophic requirements. Of 998 colonies tested, one had an additional requirement for methionine; its nutritional phenotype then was Thr$^-$Leu$^-$Thi$^-$Trp$^-$Met$^-$, and it still harbored the RP4::Tn*1861* plasmid. To discover whether the Met$^-$ auxotrophy resulted from transposition of a copy of Tn*1861* from RP4::Tn*1861* to the chromosomal *met* locus, another IncP plasmid, R751 (Tp^r), was introduced into the Met$^-$ cell carrying RP4::Tn*1861*. All R751$^+$ transconjugants (selected as Tp^r) were Thr$^-$Leu$^-$Thi$^-$Trp$^-$Met$^-$ and lacked the RP4::Tn*1861* plasmid; their resistance phenotype was Tc^{s}Cb^{s}Km^s but still Hg^r, presumably because Tn*1861* had undergone transposition to the chromosome. The frequency of reversion to Met$^+$ in these transconjugants was about 10^{-9}/cell, but three Met$^+$ revertants tested were all Hg^r. So, reversion either occurred without excision of Tn*1861* from the *met* locus or Tn*1861* did excise precisely from the *met* site and reinserted elsewhere on the chromosome without causing a new auxotrophic mutation; or there were multiple copies of Tn*1861* on the chromosome of these *E. coli* transconjugants, similar to the situation in *P. putida*.

TRANSPOSITION AND AUXOTROPHY IS INDEPENDENT OF HOST *recA* FUNCTIONS

To determine whether Tn*1861* transposition and the induction of mutations depended on host recombination functions, an R751$^-$ segregant of the Met$^-$Hg^r transconjugant (strain AC1016) was isolated. Lysates of this strain did not show the presence of any plasmid DNA band, suggesting that the mercury resistance determinant was chromosomal. This strain was then transformed to Sm^r with RSF1010 plasmid DNA and grown in nutrient broth for about 20 generations for analysis of further instability. Of 656 colonies tested, one had acquired proline auxotrophy in addition to the requirements for threonine, leucine, thiamine, tryptophan, and methionine (strain AC1018, Table 1). The frequency of reversion to Pro$^+$ was less than 10^{-9}. Thus, Tn*1861* appeared to induce multiple auxotrophy within the same cell line, presumably indicating the presence of multiple copies on the chromosome. When the RSF1010 plasmid DNA isolated from this Thr$^-$Leu$^-$Thi$^-$Trp$^-$Met$^-$Pro$^-$ mutant was used to transform *E. coli* cells treated with $CaCl_2$, Sm^r transformants were produced and a small proportion of these (0.8%) had also acquired mercury resistance. Thus, Tn*1861* could undergo transposition in *recA* cells from the chromosomal site onto plasmids and from plasmids onto other chromosomal locations to induce additional auxotrophy.

PHYSICAL CHARACTERIZATION OF THE TRANSPOSON

DNA isolated from strain AC1020, a transformant carrying RP4::Tn*1861*, was subjected to restriction endonuclease digestion and electrophoresis on agarose gels. Analysis of the gels (Figure 1) suggested that, by comparison to RP4, RP4::Tn*1861* had four EcoRI sites, two HindIII sites, and one site sensitive to BamHI. Since RP4 has single sites sensitive to EcoRI, HindIII, and BamHI, the Tn*1861* portion of RP4::Tn*1861* apparently contained three EcoRI sites, one HindIII site, and no site sensitive to cleavage by BamHI.

As shown in Table 4, isolation of RSF1010 plasmid DNA from *E. coli* cells without Tn*1861* and its electron microscopic examination showed the presence of a single size class of covalently closed circular DNA with a molecular mass of about 5.5 million daltons (Mdal). Examination of RSF1010 DNA isolated from mercury-resistant multiply-auxotrophic cells of *E. coli* and *P. putida* also showed the presence of a second type of covalently-closed circular (CCC) DNA with a molecular weight of 10.7 Mdal.

Most RP4::Tn*1861* plasmids isolated from AC1020 had a molecular weight of 43.5 Mdal compared with RP4 plasmid DNA which has a mean molecular weight around 38 Mdal. However, one exception was noted: approximately 10% of the CCC DNA

Table 4. Molecular Weights of Plasmid DNA Isolated from Hg^r and Hg^s Strains

Plasmid	*Number CCC DNA molecules measured*	*Mol. wt. x 10^6*
RSF1010	*10*	*5.5 ± 0.2*
*RSF1010::Tn*1861	*82*	*10.7 ± 0.3 (15%)*
		5.5 ± 0.2 (85%)
RP4	*5*	*38.0 ± 1.0*
*RP4::Tn*1861	*20*	*43.5 ± 1.4 (90%)*
		49.6 (10%)

molecules measured were 49.6 Mdal, suggesting that in these molecules, RP4 had possibly acquired two copies of Tn*1861*.

On the basis of contour length measurements of CCC plasmid DNA isolated from both Hg^s and Hg^r cells, we concluded that the molecular size of Tn*1861* was around 5.2 (±0.3) x 10^6 daltons.

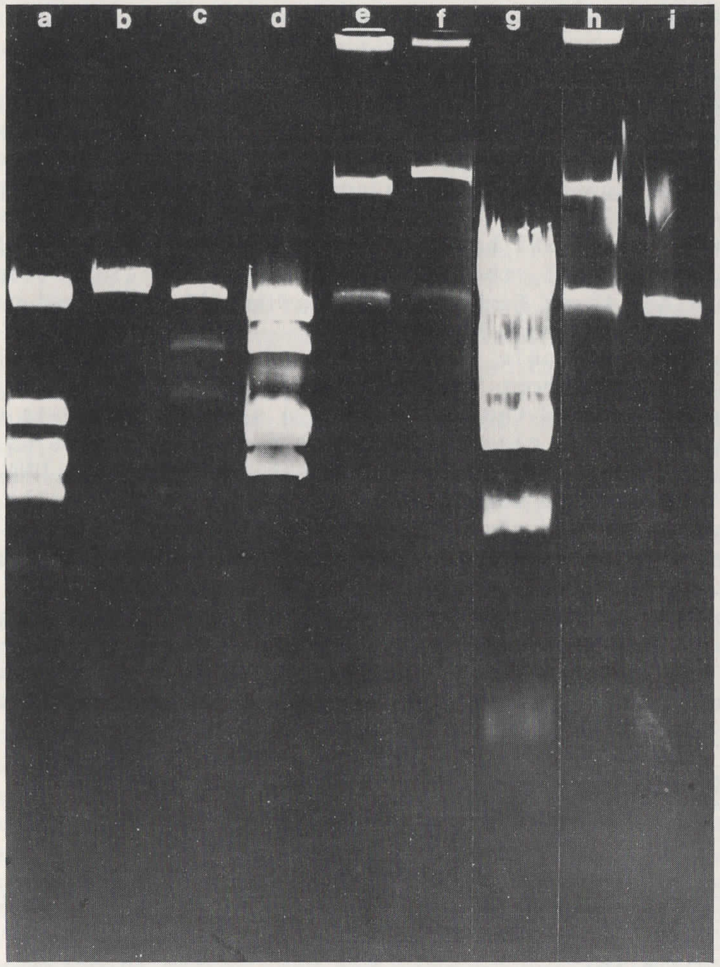

*Figure 1. Agarose slab gel electrogram of plasmid DNA digested with restriction endonucleases. From left to right: EcoRI digests of (a) λ, (b) RP4, (c) RP4::Tn*1861*; BamHI digests of (d) λ, (e) RP4, (f) RP4::Tn*1861*; HindIII digests of (g) λ, (h) RP4, (i) RP4::Tn*1861.

CONCLUDING REMARKS

The translocation of the mercury resistance gene(s) in Tn*1861* from the chromosome of *P. putida* to plasmids, including such broad-host range plasmids as RP4, and the subsequent transposition of Tn*1861* to the chromosome of *E. coli* is reminiscent of that reported for Tn*501*, a transposon also specifying mercury resistance.

Several similarities between Tn*501* and Tn*1861* can be cited. Like Tn*1861*, Tn*501* was characterized initially as part of a non-conjugative plasmid, pVSI, in *P. aeruginosa* (14). Additionally, Tn*501* is a discrete 5.2 Mdal DNA segment that can undergo transposition from one plasmid to another in absence of host recombination functions (1). The enzyme responsible for converting mercuric ions to volatile metallic mercury is mercuric reductase and data from the laboratory of S. Silver have demonstrated the immunological similarity between the mercuric reductases specified by Tn*501*, Tn*1861* and other plasmids conferring mercury resistance.

Further, restriction endonuclease analysis of RP4::Tn*1861* plasmid DNA revealed that the Tn*1861* portion contained three EcoRI sites and one HindIII site. Similarly, Tn*501* apparently contains three EcoRI sites and a single site sensitive to HindIII (1). Also, multiple copies of Tn*1861* occur on the RP4 plasmid, as found with the RP4::Tn*501* plasmid (1).

The only difference between Tn*501* and Tn*1861* appears to be the continuing genetic instability of both *P. putida* and *E. coli* cells harboring Tn*1861*, leading to multiple auxotrophy.

Such a comparison seems to suggest that, like the β-lactamase specified by Tn*A* (11), the rapid spread of mercury resistance in nature might have been caused by the *recA*-independent transposition of the mercury resistance (Tn*501*, Tn*1861*) gene(s). Although the extent of genetic homology between Tn*1861* and Tn*501* has not been determined, the antigenic similarity of the gene product, mercuric reductase; the mutagenic potential; the physical evidence with respect to the size of the DNA segment; and the number of sites sensitive to EcoRI and HindIII; all point to such a possibility.

Since heteroduplex analysis revealed that Tn*501* is bounded by small, inverted repeats of approximately 150 base pairs (1), it would be interesting to determine whether Tn*1861* also displays this characteristic.

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DISCUSSION

Shapiro: When you get these auxotrophic mutations presumably caused by insertion of a mercury transposon, can you transduce another strain to mercury resistance and then recover the auxotroph? Because even if you have several transposon copies so that revertants keep resistance, doing it the other way around you should see linkage between the mercury resistance and the auxotrophic mutation. Have you done that?

FRIELLO: No, we haven't done the transduction experiments, but we have done interrupted mating experiments and have found an association between markers that were transferred and the level of mercury resistance. We used AC1000 as the donor and mapped different parts of the chromosome. For markers at various places on the chromosome we found 2 to 35% of the cells that have received the marker have also received mercury resistance simultaneously. So through conjugation we've found that there is co-transfer. Whether there is a co-transduction or not, I don't know. I assume there would be.

Novick: I have one peripheral comment to make. In listening to this and other work on transposons I'm struck by the disservice that has been paid to a group that discovered a phenomenon almost identical to this in 1963 and 1964. I'm referring to Dawson and Smith-Keary who described unstable proline auxotrophy in *Salmonella* (1, and ref. 9 above). They didn't quite understand what the phenomenon was and people who read the papers in those days didn't understand it either. But it seems to me in retrospect that they really had their hands on a mobile transposable element that was inserting into the proline genes causing auxotrophy that would revert, and they would pick up other auxotrophs in the same strain at the time of proline reversion. They didn't have any marker on this thing at that time and whether there was a resistance gene on it or not we may never know. However, I feel at this point that one might attribute to them the first description of transposons in bacteria.

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HITCHHIKING TRANSPOSONS: A NEW CLASS OF GENETIC ELEMENT IN *STAPHYLOCOCCUS AUREUS*

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This paper provides a conceptual framework for the investigation of mobile genetic elements that encode some of the more unusual staphylococcal products. The mobility of these elements is manifested most commonly by co-transfer with other known genetic elements such as plasmids or phages. However, stable linkage is not established. A typical example is co-transduction of methicillin resistance (Mc^r) and enterotoxin B production (SEB) with either of two unrelated plasmids. Following such transfer, the plasmid is unchanged and the Mc^r-SEB determinant occupies its usual chromosomal site.

We propose that such determinants are repressor-controlled transposons, that have a primary chromosomal attachment site where they are maintained by a repressor and from which they excise during vegetative phage growth, either through their own excision enzyme(s) or through those provided by a phage. They may then insert with variable frequency into a secondary insertion site on a carrier replicon - either the phage itself or a plasmid. Such complexes are encapsulated and transferred by the phage to a new host where, in the absence of repressor, they excise from the carrier replicon and re-insert into the primary chromosomal site. Evidence will be presented in support of this model, in connection with our studies of Tn*554*, a prototype of these elements.

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PLASMIDS IN CYANOBACTERIA[1]

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The cyanobacteria (blue-green algae) are Gram-negative, oxygen-evolving, photosynthetic eubacteria. As a group, they are both ancient and diverse, first appearing in the fossil record some 2.5 to 3.0 billion years ago, and now showing a range of chromosomal G+C contents (from 35 to 71 mol %) nearly as great as that shown by prokaryotes as a whole (12,15). They were important in the past because it was through their efforts that the earth's atmosphere turned from anaerobic to aerobic and because it was from them (or their near kin) that plastids, the photosynthetic organelles of eukaryotic algae and higher plants, evolved (2,9). They are important now because of their contribution to primary productivity and nitrogen fixation in a variety of habitats, and because of the potential for their exploitation as producers of usable chemical energy (in the form of H_2) at the expense of sunlight (4,7).

THE EXISTENCE OF CYANOBACTERIAL PLASMIDS

Cyanobacteria have plasmids. The first covalently-closed circular (CCC) DNAs described were from the common unicellular laboratory strains *Synechococcus* 6301 (1) and *Synechococcus*

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PR-6 (11). The subsequent screening of some thirty strains by us, and at least that many more by Simon (13) and van den Hondel et al. (18) revealed that about one-half of all cyanobacteria harbor one or more (up to eight) distinct CCC DNAs detectable by agarose gel electrophoresis of "cleared lysates" or of more purified material taken from ethidium bromide-cesium chloride gradients. Plasmid bearing strains are found in both major groups of cyanobacteria (unicellular and filamentous), and in stocks long maintained in the laboratory as well as in strains recently isolated from natural waters.

Figure 1 shows some of the strains (most of which represent different species) in which CCC DNAs were detected, and indicates the approximate molecular weights of those DNAs.

THE 30 MD - 5 MD PLASMID PAIR: EVIDENCE FOR INTERSPECIFIC TRANSMISSION

We would first like to call attention to plasmids borne by the four strains designated *Synechococcus* 6301, *Synechococcus c.* (for *cedrorum*), *Synechococcus* 6707 and *Synechococcus* 6311 in Figure 1. The nomenclature is confusing and reflects the confused (but improving [10]) state of cyanobacterial systematics. These four strains are likely to be members of different species and, in the case of *Synechococcus* 6707, different genera. This last strain shows a chromosomal G+C content of 67.7 mol %, while the others exhibit values in the range of 55 to 56 mol % (16). We (8) have shown that *Synechococcus* 6301 and *S. cedrorum* both harbor two plasmids, of approximately 30 and 5 million daltons. Figure 2 is an electron micrograph showing both. The two CCC DNAs differ in G+C content from each other (60.2 vs 53.1 mol %) and from chromosomal DNA. They produce completely different restriction-endonuclease digestion patterns (five enzymes) and show no sequence homology in DNA:DNA "Southern" hybridization experiments. Plasmids of these same molecular weights were reported by van den Hondel et al. (18) for *Synechococcus* 6311 and *Synechococcus* 6707. Restriction endonuclease digestions (with at least five enzymes) show all four 30 megadaltons (Md) plasmids to be identical, and all four 5 Md plasmids to be identical (8,18). This degree of homology between plasmids in strains whose chromosomal DNAs have diverged in G+C by more than 12% must surely mean that plasmids can be transferred between cyanobacterial species (if not indeed between cyanobacterial genera).

The 5 Md plasmid can be lost from *Synechococcus* 6301. Such loss has occurred once spontaneously and once as a result

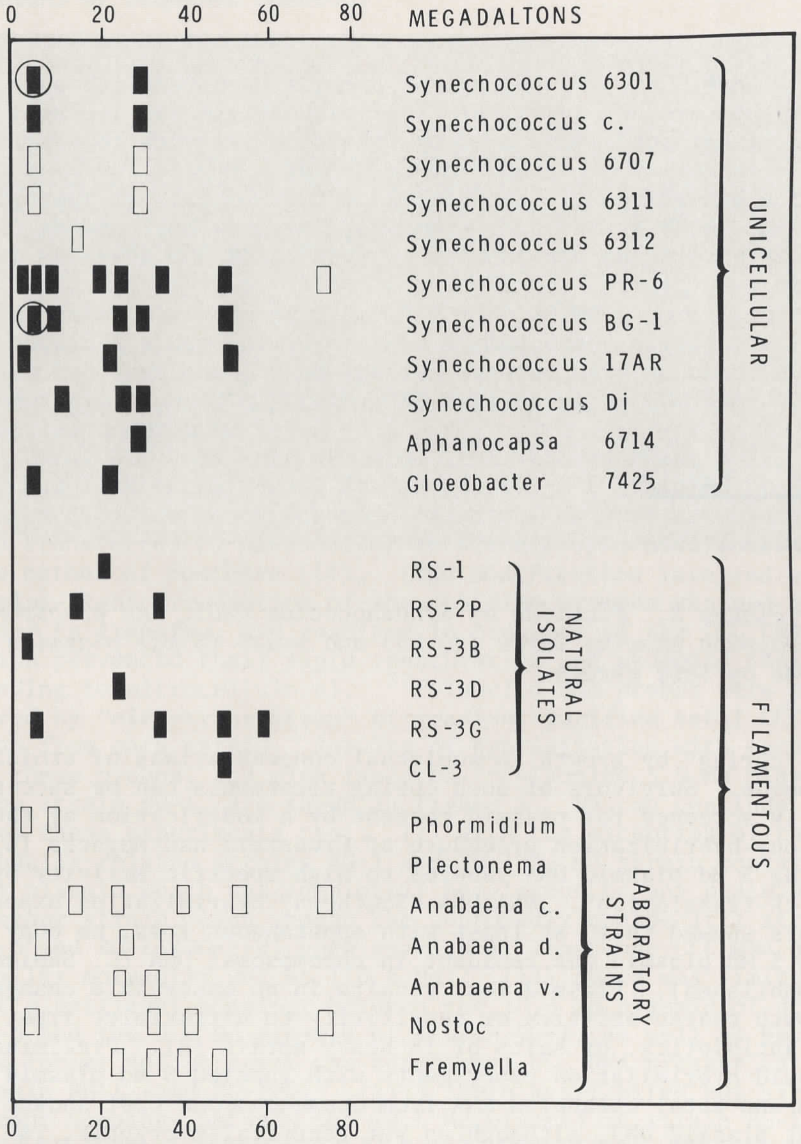

Figure 1. Distribution of plasmids in cyanobacteria. Some cyanobacterial strains which contain plasmids are shown, together with the numbers and approximate molecular weights (in millions of daltons) of these plasmids. Black bands identify plasmids whose existence was first noted or subsequently confirmed by us, white bands those seen only by others (13,18), and circled bands indicate plasmids whose spontaneous or induced loss was observed by us.

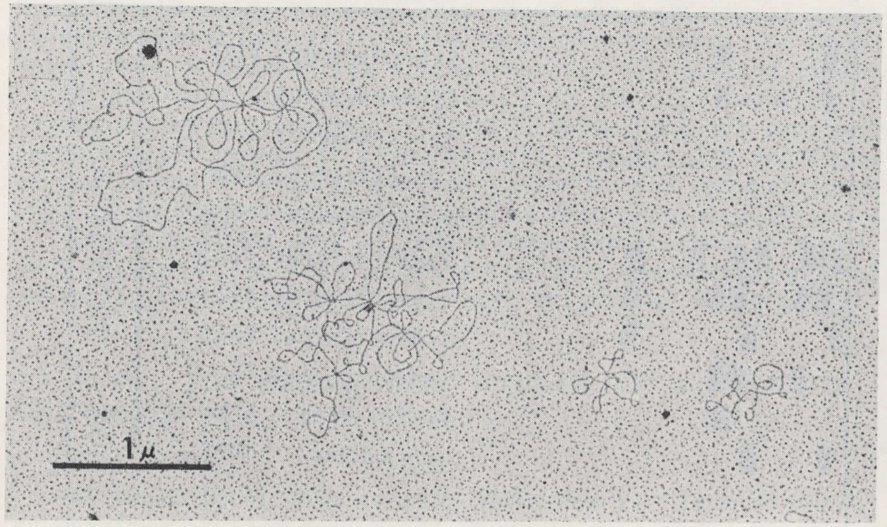

Figure 2. Plasmids of Synechococcus *6301. An electron micrograph showing large (30 Md) and small (5 Md) plasmids borne by this strain.*

of "curing" by growth in sublethal concentrations of ethidium bromide. Survivors of such curing treatments can be successfully screened for plasmid content by a modification of the colony-hybridization procedure of Grunstein and Hogness (6), using 5 Md plasmid DNA labeled to high specific activity by "nick-translation". DNA:DNA "Southern" hybridization experiments showed that, at least with spontaneous loss, no copy of the 5 Md plasmid was resident in chromosomal DNA (C. Sapienza, unpublished). Plasmid loss results in no observable change in growth characteristics or sensitivity to ultraviolet light, 14 antibiotics, or salts of 15 heavy metals (8). Preliminary liquid hybridization experiments with labeled 5 Md plasmid DNA and total unlabeled RNA from *Synechococcus* 6301 showed that plasmid DNA, although as yet genetically cryptic, is extensively transcribed *in vivo*.

PLASMIDS IN MARINE UNICELLS: EVIDENCE FOR (TRANSPOSABLE?) REGIONS OF SEQUENCE HOMOLOGY

The *Synechococcus* strains designated PR-6, BG-1 and 17AR in Figure 1 are marine unicells. The first two are separate isolates of what is considered to be a single species (*Agmenellum quadruplicatum*). The third is thought to represent a different species (*Coccochloris elabens*). Roberts and Koths (11) showed (and we have confirmed) that strain PR-6 contains five to seven CCC DNAs; here we concentrate on major plasmids of approximately 3.5, 10.6, 26 and $\geq$50 Md. Strain BG-1 contains major plasmids of 5.9, 10.6 and $\geq$50 Md. Fortuitously we obtained a derivative of this strain, here called BG-1FD, which had lost the 5.9 Md species (again, with no observable phenotypic change). Strain 17AR carries major CCC DNAs of 1.85, 33 and $\geq$50 Md.

Total CCC DNAs were prepared from strains PR-6, BG-1, BG-1FD and 17AR (with pBR322 DNA as control) by ethidium bromide-cesium chloride centrifugation, resolved on 0.7% agarose gels, and transferred to nitrocellulose filters by a modification of the method of Southern (14). (The modification involved ultraviolet-light irradiation of the ethidium bromide stained gel prior to transfer, and resulted in cleavage of the CCC DNAs which prevented their rapid renaturation and promoted their binding to nitrocellulose). ^{32}P-labeled DNA probes were prepared by "nick-translation" of gradient-purified total CCC DNAs from each strain. Results of hybridization with each of these probes, and with isolated ^{32}P-labeled 5.9 Md plasmid from strain BG-1, are shown in Figure 3. We can conclude from these experiments that: (i) with the exception of the clonally-related strains BG-1 and BG-1FD, no strain contained any plasmid which was completely homologous to any plasmid in another strain, even though the supposedly conspecific strains PR-6 and BG-1 (BG-1FD) both carried at least one CCC DNA of similar molecular weight (10.6 Md); (ii) there was limited but significant homology between the 10.6 Md plasmid of strain PR-6 and the 5.9 Md plasmid of strain BG-1, as well as between the former and at least one additional CCC DNA present in strain BG-1 (and BG-1FD); (iii) there was limited but significant homology between the 10.6 Md plasmid in strain BG-1 (and BG-1FD) and some plasmid or plasmids (other than the 3.5 Md species) present in strain PR-6; (iv) there was limited but significant homology between higher molecular weight DNAs present in each strain (PR-6, BG-1, BG-1FD and 17AR) and some plasmid(s) in each other strain; (v) the 5.9 Md plasmid of strain BG-1 showed homology with the 10.6 Md and higher molecular weight plasmids in both strains BG-1 (and BG-1FD) and PR-6

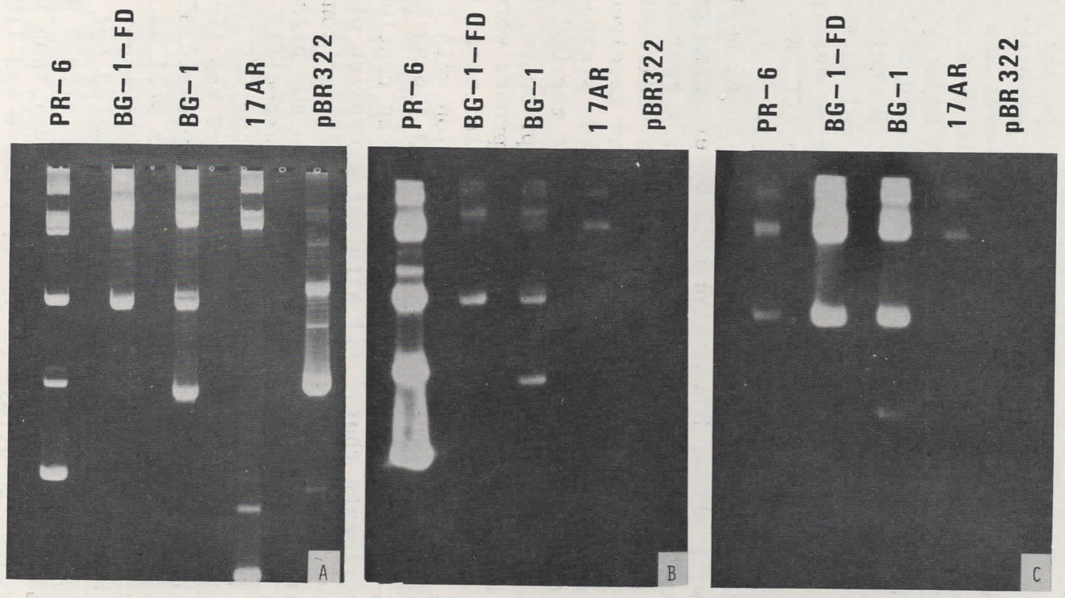
PR-6
BG-1-FD
BG-1
17AR
pBR322
A
B
C

Figure 3

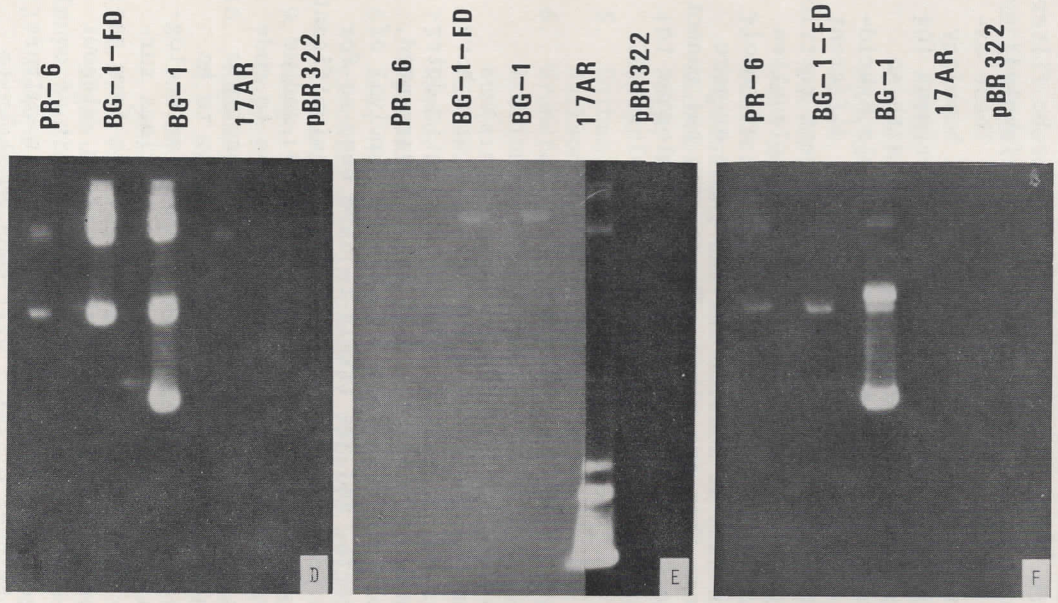
PR-6
BG-1-FD
BG-1
17AR
pBR322
D
E
F

Figure 3. Homologies between plasmids in marine unicellular cyanobacteria. A: an agarose gel of total CCC DNAs from strains PR-6, BG-1FD, BG-1 and 17AR, with pBR322 DNA as control. Panels B-F: results of "Southern" hybridization to DNAs transferred to nitrocellulose from gels like that shown in panel A, with (B) total PR-6 CCC DNA as labelled probe, (C) total BG-1FD CCC DNA as probe, (D) total BG-1 CCC DNA as probe, (E) total 17AR CCC DNA as probe, or (F) purified 5.9 Md BG-1 plasmid as probe.

(but not 17AR); and (vi) the 3.5 Md plasmid of strain PR-6 and the 1.85 Md plasmid of strain 17AR showed no detectable homology with any plasmid in any other strain.

In further "Southern" hybridization experiments (not shown), purified 10.6 Md plasmids from strains PR-6 and BG-1FD, 3.5 Md plasmid from strain PR-6, and 5.9 Md plasmid from strain BG-1 were labeled with ^{32}P and used separately to probe filter-bound *Hae* III restriction fragments of purified (unlabeled) preparations of each of these individual plasmids. These experiments showed that: (i) regions of interplasmid homology were not always confined to a single restriction fragment (or to a pair of possibly adjacent fragments) although with no plasmid did *all* restriction fragments give detectable hybridization signals with heterologous probes; and (ii) different probes could give hybridization signals with different restriction fragments of a given plasmid. The simplest explanation for these data is that there is more than one class of homologous elements, and that some plasmids can bear one or more representatives of more than one class. We assume, but cannot yet confirm, that these regions of homology are analogous to the transposable elements borne by many bacterial plasmids.

WHAT ARE CYANOBACTERIAL PLASMIDS FOR?

Neither we nor anybody else knows why cyanobacteria have plasmids. The suggestion that they might bear genetic determinants for photosynthetic functions (11) seems unwarranted, given that these functions are essential for the survival of almost all cyanobacteria, and are likely to have been so for at least 2.5 billion years (12). We have observed significant variations in heavy-metal and antibiotic resistance, and we suspect that some cyanobacterial plasmids will prove to code for such resistances. Two other cyanobacterial phenotypic characteristics seem likely, from indirect evidence, to be plasmid-determined. The first is gas-vacuolation, an ecologically important trait involved in formation of noxious surface blooms. Walsby (17) showed that the ability to form gas vacuoles can be irreversibly lost at high frequency, without mutagenesis. The second is toxin production, a trait of equal ecological importance, which is also readily lost in culture (5). We have no doubt that plasmids will soon be shown to play an important role in cyanobacterial distribution and population dynamics. We also expect that readily curable plasmids, like the 5 Md plasmid of *Synechococcus* 6301 and the 5.9 Md plasmid of *Synechococcus* BG-1, will prove useful as self-cloning vehicles for the further development of cyanobacterial molecular biology, a field still in its infancy (3).

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DISCUSSION

Shapiro: Can I make a comment and a question? The comment is that in Bob Haselkorn's lab in Chicago they've shown that the *nif* DNA in one of the blue-green algae, I think it's *Nostoc,* is homologous with the *nif* region of *Klebsiella.* This is in agreement with the idea that this is a very strongly conserved region which has moved around perhaps by transposition between many Gram-negative organisms. If these are Gram-negative strains, can you introduce plasmids either by transformation or by conjugation from other Gram-negative bacteria, in particular things like RP1?

DOOLITTLE: We've tried RP4, by growing donor and recipient strains together, although there's no growth condition under which both *E. coli* and blue-greens are happy, and that didn't work; and we've tried transforming it in, assuming that we could use the normal calcium chloride cold shock treatment which one uses with *E. coli* on blue-greens. They do survive that treatment, but we got no RP4 transformants. We tried putting pBR322 into it - I guess that's illegal now - fortunately it didn't work. That's about as far as we've gone on this. I think it may be possible.

Barnsley: Are there any known biochemical functions in filamentous bacteria in the wild that don't occur in your lab stocks? I was just struck by the absence of (some) plasmids from your lab stocks in the slide which you showed.

DOOLITTLE: Yes, I think, in general it's probably reasonably easy to lose plasmids from laboratory stocks and that's one of the reasons we went to the wild - out in Dartmouth - and got muddy looking strains. But not being able to identify those strains I couldn't say whether they differ from our lab stocks of the same species - I mean they're probably not the same species.

Lederberg: Have you collected chloroplast-minus mutations in your strains?

DOOLITTLE: Blue-green algae don't have chloroplasts. They are chloroplasts.

Lederberg: I mean pigmentless variants so that you can start supplying something exogenously as has been done with algae, and perhaps build up a genetic scheme, etc.

DOOLITTLE: Well, what we're hoping to do is use the small plasmid as a self-cloning vehicle so that we could phenotypically revert mutants in our strain by introducing the small plasmid with a piece of chromosomal DNA from wild-type into the mutant strain.

Lederberg: With more markers?

DOOLITTLE: Yes.

Lederberg: I think this is an easy way to go because you can always pick out white colonies.

DOOLITTLE: Well, pigment markers are rare but obvious, yes.

Unidentified: Could it be that a few in the population will have to grow on more complex media?

DOOLITTLE: No, they won't.

INDUCIBLE TRANSPOSITION OF Tn*917* IN *Streptococcus faecalis* STRAIN DS16[1]

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A drug resistant clinical isolate of *Streptococcus faecalis* subspecies *zymogenes*, DS16, is currently under investigation in our laboratory (1,6,7). It harbors two plasmids, each present at 1-2 copies per chromosomal genome equivalent. One plasmid, pAD1, is a 35 Mdal conjugative plasmid which, in addition to coding for transfer functions, codes for bacteriocin (bac) and hemolysin (hem) activities. The other plasmid, pAD2, is nonconjugative, has a mass of 15 Mdal, and encodes resistance to streptomycin (Sm), kanamycin (Km), and erythromycin (Em). The Em determinant belongs to the MLS class of resistance (8) and is inducible. The MIC for all three antibiotics is greater than 2 mg/ml. In addition to these plasmid-determined resistances, DS16 also has a chromosome-borne tetracycline resistance determinant.

The transfer of pAD1 from DS16 into the plasmid-free

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Streptococcus faecalis strain JH2-2 occurs at a frequency of approximately 10^{-3} to 10^{-2} per donor, and it can mobilize the nonconjugative pAD2. Nonhemolytic resistant transconjugants appear at a frequency of about 10^{-9} and generally have pAD2 as the sole plasmid; resistant transconjugants, which are also hemolytic, contain a single plasmid of 50 Mdal, representing a cointegrate of pAD1 and pAD2 (7). These appear with a frequency of approximately 10^{-8} to 10^{-7}.

In addition, another class of transconjugants is obtained in which only the Em determinant of pAD2 and the hem-bac activities are expressed. Isolation and characterization of the plasmid DNA from these transconjugants has revealed a single 38 Mdal circular molecule. This plasmid was self-transmissible at high frequency (equivalent to pAD1). Furthermore this plasmid is stable upon subsequent transfer to new recipients. These and other data have demonstrated that the Em determinant resides on a 3.3 Mdal transposable element which we have designated Tn*917* (6). Tn*917* has inverted repeats (280 base pairs) flanking the resistance determinant.

Considering the inducibility of the Em resistance determinant (6) along with the reported evidence that transposons code for proteins involved in transposition (4), we tested the possibility that transposition could be induced by pregrowth of cells in low (inducing) concentrations of Em; i.e., proteins involved in both transposition and erythromycin resistance may be under a common control system. Prior to mating with the plasmid-free strain JH2-2, strain DS16 was grown for 4 hours in the presence or absence of 0.5 µg/ml of Em. Hemolytic Em resistant transconjugants were then selected. The results of 3 separate experiments are shown in Table 1. Without pregrowth in Em, donor cells showed little or no formation of pAD1::Tn*917*; however, donor cells pregrown in Em exhibited a significant increase. Subsequent experiments (e.g., see Figure 1) have, in general, exhibited a ten- to twenty-fold increase in transposition. Thus transposition can be enhanced by pregrowth in low levels of Em.

The observation that transposition can be induced suggests that inhibitors of RNA and protein synthesis would inhibit transposition. Furthermore, a model recently proposed by Shapiro (5) postulates the need for DNA synthesis in the transposition process. We thus looked at the effect of inhibitors of RNA, protein and DNA synthesis, namely rifampicin (rif), chloramphenicol (CM), and *p*-hydroxyphenylazouracil (hpUra) respectively. The latter drug is an effective inhibitor of DNA synthesis in Gram-positive bacteria (2). Strain DS16 was grown in 0.5 µg/ml of Em and samples were removed and exposed to inhibitors for different lengths of time. The cells were then washed free of drug, resuspended in fresh medium at the original volume, and immediately mated with strain JH2-2 for 4 hours. After mating hemolytic Em-resistant transconjugants were selected and screened for their susceptibility to Km and Sm. Those transconjugants sensitive to Km and Sm but resistant to Em were recorded as carrying pAD1::Tn917. The amount of transposition is represented as the percentage of Em^r transconjugants that were Sm^s Km^s.

Table 1. Erythromycin Enhanced Transposition[a]

Donor treatment	*Hem*$^+$ *Em*^{r} *transconjugants per donor*[b]	*Frequency of pAD1::Tn917 cells*[c]
No pregrowth in Em	3.6×10^{-8}	3.8%
4 hr pregrowth in Em	10×10^{-8}	45.0%
No pregrowth in Em	9.4×10^{-8}	0%
4 hr pregrowth in Em	18.3×10^{-8}	24.0%
No pregrowth in Em	1.1×10^{-8}	0%
4 hr pregrowth in Em	6.6×10^{-8}	31.0%

[a] *Formation of pAD1::Tn917 and its transfer were measured in three different experiments.*

[b] *The procedures involved in mating and selection for transconjugants are as reported elsewhere (3).*

[c] *Based on the number of hemolytic* Em^r, Sm^s, Km^s *transconjugants divided by the total number of hemolytic* Em^r *transconjugants.*

As shown in Figure 1, in the absence of inhibitor we observed an increase in the transposition frequency as the induction time was increased. However, Rif, CM, and hpUra all brought about a cessation of transposition. The inhibition by Rif and CM implied a requirement for protein synthesis, but because of the rather long time course of the experiment and the effect of hpUra, we cannot rule out the possibility that Rif and CM were acting indirectly through an inhibition of DNA synthesis. (It is noteworthy that with CM and hpUra, cell viability remained essentially constant during drug exposure, and the frequency of transfer of Em-resistance after drug exposure was unaltered. With Rif viability decreased 1-2 logs over the course of three hours, yet the absolute number of transconjugants after drug exposure did not decrease accordingly.) With a few exceptions, most plasmids will not initiate a round of replication if protein synthesis is inhibited. Experiments have shown that in the presence of Rif or CM,

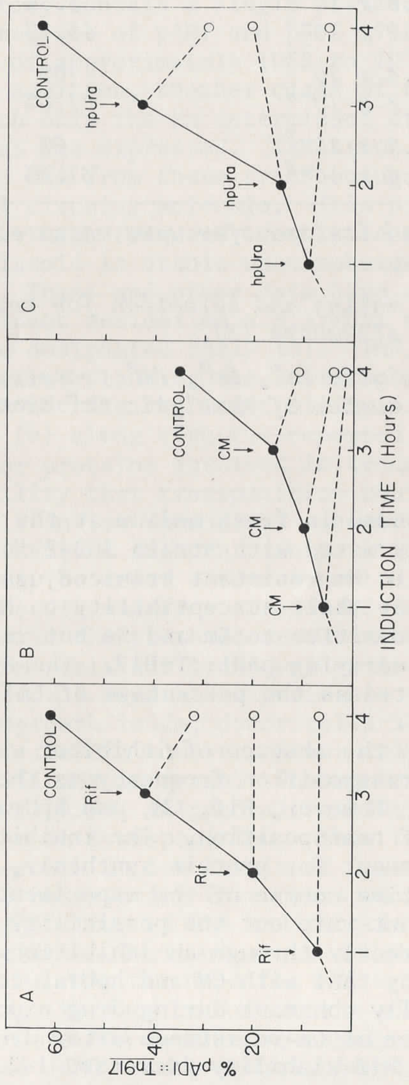

Figure 1. Effect of Rif, CM and hpUra on the induction of transposition. Arrows indicate time of addition of one of the three drugs during exposure to Em (0.5 μg/ml). Incubation time at 37°C was as indicated in the presence of: A) Rif (10 μg/ml); B) CM (50 μg/ml); or C) hpUra (500 μg/ml), such that Em was present for a total of 4 hours. After the cells were centrifuged and washed free of the drugs, DS16 was mated with JH2-2 using a donor to recipient ratio of 1:10. The open circles indicate the level of transposition at these points; the filled circles represent DS16 at the indicated time points without addition of drug. The percentage of cells carrying pAD1::Tn917 was calculated as described in Table 1.

replication of pAD1 and pAD2 will not proceed to more than one doubling. Other workers have presented evidence that transposition is controlled by certain transposon encoded gene products (4), and it appears likely that the induction of Tn*917* transposition involves enhanced expression of a "transposase". While this expression is probably blocked by Rif and CM, the indirect inhibition of DNA synthesis by these drugs masks this observation. The requirement for DNA synthesis, however, seems clear and the data are consistent with the Shapiro model (5).

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PLASMID RELATED SEX PHEROMONES IN *Streptococcus faecalis*[1]

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Recent studies in our laboratory have shown that recipient strains of *Streptococcus faecalis* excrete heat stable, protease-sensitive sex pheromones which induce donors harboring certain conjugative plasmids (see Table 1) to become adherent (4). Using various inhibitors, we found that the induction requires RNA and protein synthesis, but not DNA synthesis (4). In addition to being able to adhere to recipients, induced donor cells would also aggregate, or clump, in the absence of recipients. (It is noted that these bacteria are nonmotile and aggregation is initiated by random collision). Thus, we initially designated the pheromone produced by recipient cells as "clumping-inducing agent" (CIA). Using a microtiter assay

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Table 1. Relevant Plasmids Used in this Study

Plasmid	Mol. Weight	Related phenotype[a]	Source
pAMγ1	3.5×10^7	Hemolysin, conjugative	DS-5C1
pPD1	3.5×10^7	Hemolysin, conjugative	39-5
pOB1	4.6×10^7	Hemolysin, conjugative	5952
pAD1	3.5×10^7	Hemolysin, conjugative	DS16
pAD1::Tn917	3.8×10^7	Hemolysin, Erythromycin res., conjugative	DS16
pAMα1	6.0×10^6	Tetracycline res, nonconjugative	DS-5C1

[a] *In our hands hemolysin activity is always accompanied by bacteriocin activity. Brock and Davie (1) and Granato and Jackson (6) have shown that the hemolysin and bacteriocin activities produced by* zymogenes *subspecies are probably one and the same protein.*

Table 2. Mating Enhancement Activity of Filtrates Taken From Late Log Phase Cultures of OG1-10 and JH2-2[a]

Mating[a]	Filtrate used for 45' pretreatment of donors	Transconjugants per donor after 10' mating
OG1-12 (pAMγ1, pAMα1) x OG1-RF1	control (no filtrate)	$<4.0 \times 10^{-7}$
	OG1-10 (plasmid free)	6.0×10^{-4}
	JH2-2 (plasmid free)	4.0×10^{-3}
OG1-50 (pAD1::Tn917) x OG1-RF1	control (no filtrate)	4.0×10^{-7}
	OG1-10 (plasmid free)	3.0×10^{-1}
	JH2-2 (plasmid free)	1.0×10^{-1}
39-5Sα (pPD1, pAMα1) x OG1-RF1	control (no filtrate)	$<5.0 \times 10^{-7}$
	OG1-10 (plasmid free)	2.0×10^{-3}
	JH2-2 (plasmid free)	2.0×10^{-3}

[a] *The specific conjugative plasmid of the donor is indicated in parenthesis. Where a selectable marker was lacking (e.g., for pAMγ1 and pPD1) the transferability of the conjugative plasmid was measured indirectly through its ability to mobilize the selectable non-conjugative pAMγ1. Matings were as previously described (3).*

system (5) and serial 2 fold dilutions we have been able to quantitate the amount of CIA activity in Millipore filtrates of recipient cultures. This technique is also useful in determining the relative sensitivities of different responding donors to a single recipient filtrate.

If donor cells are mixed (mated) with appropriate recipients in broth for the relatively short period of 10 minutes, the number of resulting transconjugants is very few, generally 10^{-7} per donor or less. However, if prior to mating the donor cells are exposed to a recipient filtrate for 45 minutes, the number of transconjugants increases by 3-6 orders of magnitude. Examples of this are shown in Table 2.

Subsequent studies have shown that recipient strains actually produce multiple CIA activities. Table 3 shows the response of four different clinical isolates, each harboring a different conjugative plasmid, to filtrates of JH2-2 hosts containing, in each case, a different conjugative plasmid. When strain 5952 (harboring the conjugative plasmid pOB1) was exposed to a filtrate of the plasmid-free strain JH2-2, it underwent a clumping response, with CIA showing a titer of 8. When exposed to a filtrate of the same strain, but now harboring pOB1, there was no response. This was expected since this was a homologous system, and the plasmid always shuts off the production of endogenous CIA in its host. However, when JH2-2 harbored any of the other three plasmids, there was full expression of CIA activity. For strain 39-5, which harbored the conjugative plasmid pPD1, the filtrate of the plasmid-free strain had a CIA titer of 64. Again the only failure to produce CIA was when the host harbored the homologous plasmid pPD1; although some reduction of activity was observed when harboring plasmid pAMγ1. For strain DS-5C1, where pAMγ1 was the conjugative plasmid, the filtrate of the host harboring pAMγ1 failed to contain CIA activity as expected; however, there was also undetectable activity when pPD1 was present. Thus there may be some relationship between pPD1 and pAMγ1. In strain DS16, which harbors the conjugative pAD1, we failed to observe a response to any of the filtrates. However, while JH2-2 filtrates contained no detectable activity, filtrates of another plasmid-free strain, OG1, contained activity that was readily detectable (not shown). Also, DS16 strains which were cured of pAD1 produced a CIA activity to which the DS16 parent responded (5). It is noteworthy that when JH2-2 harbored pAD1 (Table 3), there was no effect on the production of the other CIA activities. These data indicate that recipients actually produce multiple CIA and while the acquisition of a plasmid by that recipient caused the production of the related CIA to stop, the production of other activities which are specific for different classes of plasmid donors continued.

A given CIA then is defined by the host-plasmid system being used to resolve it. We now identify these different CIA's by relating them to the plasmid originally used in

Table 3. Evidence for Multiple CIAs: Response of Clinical Isolates to Different JH2-2 Hosts Carrying Different Conjugative Plasmids

Responder strain[b] (plasmid content)	Filtrates of JH2-2 hosts containing different plasmids[a]				
	JH2-2 (plasmid free)	GD60 (pOB1)	GD50 (pPD1, pPD4)	HD1 (pAMγ1)	GD70 (pAD1)
5952 (pOB1, pOB2)	8	-	8	8	8
39-5 (pPD1, pPD2, pPD3, pPD4)	64	64	-	4	64
DS-5C1 (pAMγ1, pAMα1)	8	8	-	-	8
DS16 (pAD1, pAD2)	-	-	-	-	-

[a] The number indicates the CIA titer, i.e., the maximum dilution of filtrates to which the specific responder would undergo a clumping response.

[b] While the responders harbor 2 or more plasmids, the first indicated represents the only conjugative plasmid present.

Table 4. Examination of 100 Clinical Isolates for Response to and Production of CIA

	Sensitive to drugs	Resistant to one or more drugs	Resistant to four drugs	P[a]
Clumping response to filtrate of OG1-10	8/36 (22.2%)	26/64 (40.6%)		0.048
	8/36 (22.2%)		6/11 (54.5%)	0.050
Production of CIA				
toward 39-5	20/36 (55.6%)	52/64 (81.3%)		0.006
	20/36 (55.6%)		11/11 (100.0%)	0.004
toward OG1-12	15/36 (41.7%)	45/64 (70.3%)		0.005
	15/36 (41.7%)		10/11 (90.9%)	0.004

[a] Significance was determined using Fisher's exact test for 2 x 2 contingency tables.

detecting it. Thus, cPD1 refers to the CIA to which strains harboring pPD1 respond. Similarly, we have cAMγ1, cAD1 and cOB1.

The data of Table 4 represent a survey of 100 clinical isolates examined for their response to and production of CIA activity. The strains were obtained from University Hospital (Ann Arbor) and categorized into groups corresponding to those which were: (i) sensitive to drugs, (ii) resistant to one or more drugs, and (iii) resistant to four drugs. The drugs of concern were tetracycline, erythromycin, kanamycin and streptomycin. With regard to their response to a filtrate of the plasmid-free strain OG1-10, the drug resistant strains were significantly more likely to respond. This is presumed to be due to the presence of conjugative plasmids (R-plasmids?) in these strains. The organisms were also tested for their production of two CIAs, cPD1 and cAMγ1, using appropriate responder strains. In both cases significantly more strains within the drug resistant groups excreted these CIAs compared to the drug sensitive strains.

It is worth noting that whereas the four conjugative plasmids which have been studied for their role in specific CIA responses are hemolysin plasmids, many of the strains of Table 4 responding to the OG1-10 filtrate were not hemolytic. (Of the 100 clinical isolates, only 12 were hemolytic). Thus the ability to produce hemolysin is not directly related to the ability to respond. It stands to reason then that CIA may play a role in the evolution of drug resistance and that cells which produce multiple CIAs are more likely to acquire R-plasmids.

We are currently attempting to purify and characterize CIA; preliminary studies indicate that cPD1 probably has a molecular weight of 700-800 (2). Considering their heat-stability (they can be boiled or even autoclaved) and protease-sensitivity, CIAs may represent small peptides. We have presented a model elsewhere (5) which suggests how CIA, conjugative plasmids, and mating may be related. Thus, the conjugative plasmids must have determinants which "shut off" the production of specific endogenous CIA while at the same time potentiate a response to exogenous activity.

Whereas sex pheromones are well known in fungi and higher systems, to our knowledge these are the first examples in bacteria where one mating type excretes substances which influence the physiological state of different mating types. It will be of interest to see the extent to which this occurs in other bacteria.

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GENETIC ANALYSIS OF CONJUGATION BY RP1[1]

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RP1 is the prototype of incompatibility group P plasmids that were originally found in *Pseudomonas aeruginosa*, but have now been isolated from several different bacterial genera (5). Indeed, these plasmids are of particular interest because of their very broad host range which derives from their ability to replicate and partition successfully in many different Gram-negative hosts.

Their conjugation system is also of some interest since it differs from the well-understood F system in the following ways: (a) RP1 pili (which are morphologically different from F pili and adsorb a different set of "male-specific" bacteriophages; 7,4) must recognise some component or structure common to the cell surfaces of the wide range of bacterial genera between which the plasmid can transfer; (b) the RP1 transfer system, although highly efficient in membrane filter matings where the cells are forced into contact, is about 1000-fold less efficient in liquid broth matings; and (c) wild-type IncP plasmids determine no fertility inhibition system, so that their transfer genes and consequent P-specific phage sensitivities are expressed constitutively.

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We have therefore undertaken a genetic and physical analysis of the RP1 conjugation system, aimed at determining the underlying reasons for these differences. This communication describes the isolation of transfer-deficient RP1 mutants, and of the cloned RP1 segments necessary for their genetic analysis.

ISOLATION OF RP1 *tra* MUTANTS

RP1 mutants defective in transfer were identified by screening survivors of nitrosoguanidine-treated cell populations in replica-plate matings with a suitable recipient strain of *E. coli*. Amongst the first 100 independent mutants isolated, most carried non-suppressible mutations and were resistant to the three P-specific phages PRR1 (RNA-containing), Pf3 (filamentous single-strand DNA-containing) and PR4 (lipid-containing tailed phage containing double stranded DNA). Mutants carrying amber-suppressible mutations, or retaining sensitivity to one or more P-specific phages, were deemed of particular interest, and continued screening allowed isolation of a further 30 of these.

Table 1 shows the phenotypes of a selected group of the RP1*tra* mutants, including in particular some of those carrying amber mutations suppressible in a $SuIII^+$ host. Most mutants were resistant to the three types of P-specific phages, but two examples each of mutants still sensitive to all three phages (out of a total of 29 none of which, curiously, carried an amber mutation), and of mutants sensitive only to PR4 (out of a total of 6, including two with amber mutations) are included. Many mutants also had defects in surface exclusion, though whether this was sometimes due to the polarity of a particular amber mutation has not yet been determined.

Initial attempts at complementation analysis using the conjugational transient heterozygote technique described previously for F (1) were unsuccessful, probably because of the low donor ability of RP1 compounded in the two successive broth matings required (it was not feasible to do large numbers of membrane-filter matings), and the continued expression of surface exclusion by RP1 in stationary phase or minimal medium-grown cells. P1 transductional complementation (8) was also unsuccessful in our hands, although Barth, Grinter and Bradley (2) were able to use this technique to arrange RP1*tra*::Tn7 mutations into complementation groups.

*Table 1. RP1*tra *Mutants*

	Sensitivity to			*Donor ability (%)*		*R751 surface*
Plasmid	*PRR1*	*Pf3*	*PR4*	Su^-	$SuIII^+$	*exclusion index*
RP1	*S*	*S*	*S*	*1.2*	*0.9*	*93*
pED522	*S*	*S*	*S*	$<10^{-5}$	$<10^{-4}$	*50*
pED525	*S*	*S*	*S*	$<10^{-4}$	$<10^{-4}$	*41*
pED516	*R*	*R*	*S*	$<10^{-5}$	*2.2*	*1*
pED526	*R*	*R*	*S*	$<10^{-4}$	$<10^{-4}$	*NT*
pED511	*R*	*R*	*R*	$<10^{-4}$	*0.9*	*2*
pED615	*R*	*R*	*R*	$<10^{-5}$	*0.2*	*59*
pED530	*R*	*R*	*R*	$<10^{-5}$	*2.8*	*1*
pED534	*R*	*R*	*R*	$<10^{-4}$	*0.1*	*3*
pED557	*R*	*R*	*R*	$<10^{-5}$	*1.9*	*23*
pED573	*R*	*R*	*R*	$<10^{-5}$	*7.4*	*5*
pED617	*R*	*R*	*R*	$<10^{-5}$	*5.0*	*12*
pED619	*R*	*R*	*R*	$<10^{-5}$	*0.8*	*47*

CLONING RP1 TRANSFER REGIONS

Since transient heterozygote procedures were not feasible, we turned to cloning techniques in order to overcome the incompatibility problem so that the appropriate stable heterozygotes could be constructed.

Work by Barth et al. (2) had shown that there are two transfer regions on RP4 (which is probably identical to RP1). These are labelled Tra1 and Tra2 in Figure 1. We have therefore cloned these two regions away from the RP1 incompatibility region(s) (6) onto the ColE1-based vector plasmid pBR325. pBR325 carries resistance to ampicillin, chloramphenicol and tetracycline, and there is an EcoRI site within the *cml* gene (3).

In order to accomplish cloning of the Tra1 region (cloning of the Tra2 region will not be described here), pED765 was constructed from RP1 (Figure 1). This carries an insertion of Tn7 into the *kan* gene, and has been made Tc^s by nitrosoguanidine mutagenesis. The Tra1 region thus lies between the single EcoRI sites of RP1 and Tn7. After restricting pED765 and pBR325 DNAs with EcoRI, they were mixed and ligated. Tp^r Tc^r transformants were then selected. One of these (pED799) proved to have the expected restriction pattern, but was unstable (>90% loss in an overnight broth culture). Its DNA was therefore cleaved with EcoRI, re-ligated and Tp^r Tc^r transformants again selected. Six out of eight such

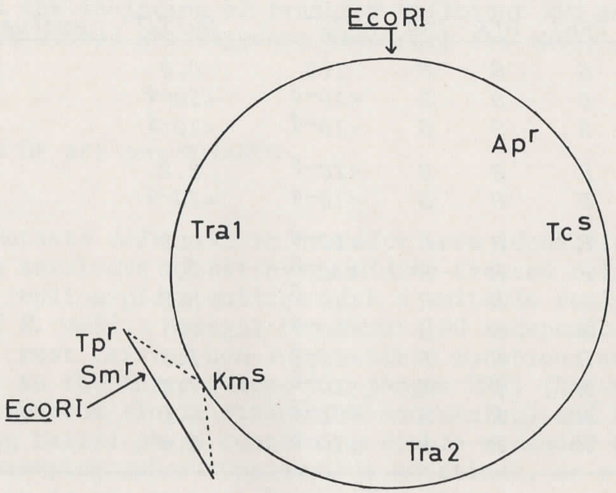

Figure 1. A map of the RP1 tet^s kan:*:Tn7 derivative pED765*

transformants (one being pED800) were now more stable (>20% loss in overnight broth culture). pED799 and pED800 gave identical EcoRI cleavage patterns, but differing *Hae*III patterns and observation of heteroduplex molecules in the electron-microscope suggested that the EcoRI fragments were joined in opposite orientations in the two plasmids (data not shown). This might affect expression of an essential replication gene.

pED800 was transfer-deficient and compatible with RP1, and the ability of RecA$^-$ host cells to transfer both plasmids independently showed that the origin of transfer, *oriT,* was included in the cloned region and is therefore located near the Tral region between the EcoRI site and the *kan* gene of RP1. pED800 was usually transferred at a several-fold higher frequency than a coexisting RP1 plasmid, probably because of its multicopy nature.

COMPLEMENTATION ANALYSIS

Stable heterozygotes carrying both an RP1*tra* mutant and pED800 were constructed either by transferring the amber-suppressible mutant from a SuIII$^+$ host into a Su$^-$ host carrying pED800, or by transforming pED800 into Su$^-$ (RP1*tra*) cells. Their quantitive donor abilities were then determined, and some representative results are shown in Table 2.

Table 2. Complementation by pED800

*RP1*tra$^-$ *plasmid*	*Sensitivity to PRR1, Pf3, PR4*	*Transfer from heterozygote (%)* RP1tra$^-$ *(Km^r)*	*pED800 (Tp^r)*
RP1	*SSS*	*1.1*	*3.1*
pED522	*SSS*	*0.1*	*0.4*
pED525	*SSS*	*0.97*	*1.6*
pED516	*RRS*	*0.28*	*1.4*
pED526	*RRS*	*0.15*	*0.8*
pED511	*RRR*	*0.32*	*2.5*
pED615	*RRR*	*0.53*	*1.5*
pED530, 534, 557, 573, 617, 619	*RRR*	*$<10^{-4}$*	*$<10^{-4}$*

All the RP1*tra* mutants so far tested that retain sensitivity to all P-specific phages (e.g., pED522 and pED525), or to PR4 alone (e.g., pED516 and pED526) were complemented by pED800. This agrees with the mapping of similar phage-sensitive RP1 *tra*::Tn7 mutants in this region by Barth et al. (2). Surprisingly, however, only two out of twenty P-specific phage-resistant amber RP1*tra* mutants were complemented, so that the majority of theRP1 *tra* genes may be located elsewhere on RP1.

Derivatives of pED800 carrying various *tra* mutations have now been obtained by homogenotisation or mutagenesis, and are being used to determine the number of genes in the Tral region (N. Hunter, L. Schmidt, and N.S. Willetts, unpublished data). So far, the mutations carried by pED522, pED516, pED511 and pED615 have been shown to be in different genes that are designated *traA*, *traB*, *traC* and *traD* respectively.

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F-DERIVED *tra*$^+$ RECOMBINANTS: TRANSFER AND TRANSPOSITION PROPERTIES[1]

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During the last 30 years a rapid dissemination amongst bacterial species of multiple resistance to antibiotics has been documented. At the molecular level this dispersion can largely be attributed to two phenomena, transposition and conjugation. Transposition is the recombination-independent translocation of DNA and provides a mechanism for the ready acquisition of drug resistance genes by different replicons. This rearranged information can be transferred between bacteria by a conjugative process either as part of, or mobilized by, a self-transmissible (conjugative) plasmid. In the proper environment, selection for drug resistance ensures the spread of such R factors.

The *Escherichia coli* fertility factor F is the archetypal conjugative plasmid and its transfer system has been the most extensively investigated. A plasmid of 100 kilobase (Kb) pairs, its transfer genes are clustered into a region of 33Kb (Fig. 1). More than 21 cistrons have been determined and most gene products have been identified by SDS-polyacrylamide gel electrophoresis (8,17) although few have been assigned definite roles in the transfer process. Exceptions are *traA* which codes for the pilin subunit (9) and *traJ* the product of which regulates transcription of the transfer operon (7,14) thus controlling the expression of more than 18 genes (Fig. 1).

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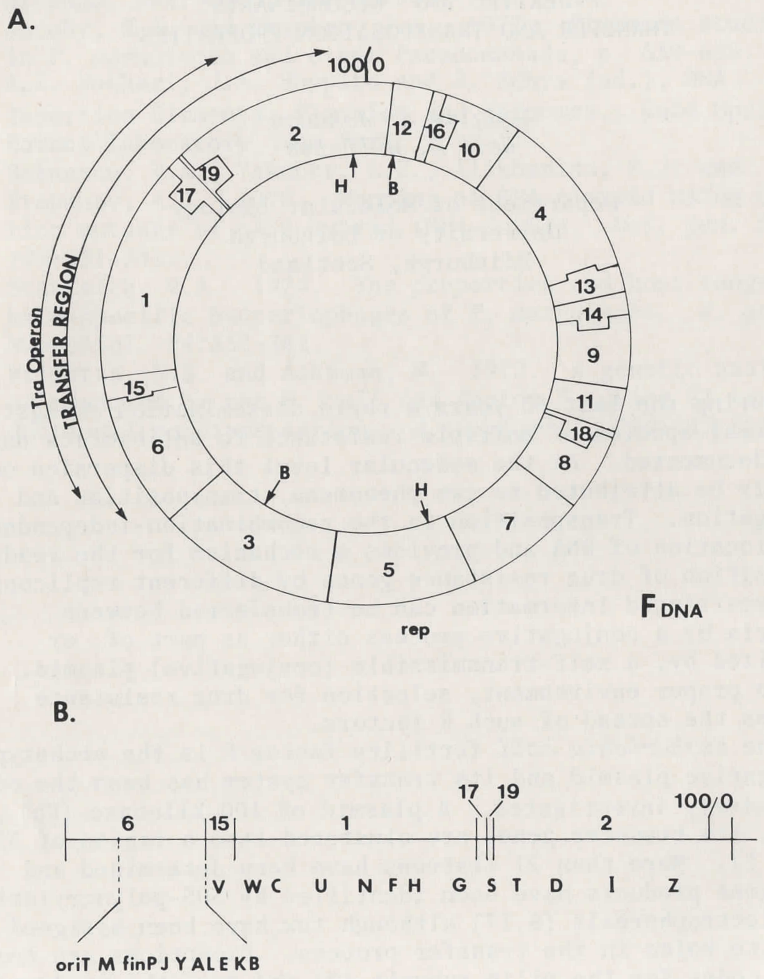

Fig. 1. (a) Physical map of F DNA based on the EcoRI fragments with the positions of the BamHI(B) and HindIII(H) sites used in the cloning experiments. Data taken from references 10, 11 and 12 was adjusted to a F size of 100 Kb (D.A. Johnson and N.S. Willetts, in preparation). (b) Location of the tra *genes within the transfer region.*

Fertility inhibition systems such as the *finO*/*finP* system (15) act to repress conjugational DNA transfer and many R factors are found to be repressed by similar mechanisms when isolated.

The organization of the *tra* genes within the transfer region has been elucidated through the analysis of λ*tra* transducing phages (16, D.A. Johnson and N.S. Willetts, in preparation) and cloned restriction fragments obtained by digestion with EcoRI (11,1) or PstI (13) or SalI and BglII (D.A. Johnson and N.S. Willetts, in preparation). As a result of these experiments the locations of most *tra* genes can be fixed within 0.5Kb (1,16, D.A. Johnson and N.S. Willetts, in preparation).

Restriction of F DNA with either BamH1 or HindIII does not lead to cleavage within the transfer region (12,10) (see Fig. 1). Thus it should be possible to clone these large fragments (44.5Kb and 55.0Kb respectively for the BamH1 and HindIII enzymes) by virtue of their conjugative properties. We have made use of this observation to isolate and characterize multicopy *tra*$^+$ recombinants. In addition the BamHI fragment has been inserted into the unique BamHI site in Tn*1* to create a new transposon which translocates both Ap^R and the F-coded conjugation system.

Porter 82

↳ 10 Tn5.

ANALYSIS OF pBR322-*tra*$_F$ RECOMBINANTS

tra$^+$ recombinants were constructed by insertion of F DNA restriction fragments into the single BamHI or HindIII site of pBR322 (D.A. Johnson and N.S. Willetts, in preparation). Transfer proficient clones were selected by their ability to transfer the Ap^R of pBR322. Unlike its parental replicon ColE1, pBR322 cannot be mobilized by the F-transfer system. Thus this protocol would not select the mini-F replicon resulting from ligation of the 55.0Kb HindIII fragment (which carries the F replication genes) although we have isolated this mini-F by a different procedure.

Restriction of the isolated plasmid DNAs, pED805 (the HindIII recombinant) and pED851 (the BamHI recombinant), confirmed the formation of the correct recombinants and revealed the orientation of the insertions. The orientation of the HindIII fragment in pED805 allowed the removal of the extraneous F DNA between the HindIII site in EcoRI fragment f5 and the BamHI site in EcoRI fragment f3 (Fig. 1) by digestion with BamHI. The recombinant pED862 contains 41.0Kb of F DNA between the BamHI site in f3 and the HindIII site in f2.

These plasmids were analysed for genes not related to transfer and as expected (11,1) none carried *pif*, resistance

TABLE I. Properties of pBR322-tra$_F$ Derivatives

	*F*lac	*pED805*	*pED851*	*pED862*
Cloning enzyme	-	*HindIII*	*BamI*	*HindIII + BamI*
Copy Number (pBR322 = 61)	*1*	*41*	*40*	*26*
Donor ability (%)	*150*	*630*	*1,400*	*250*
Surface exclusion index	*450*	*26,000*	*59,000*	*2,900*
R100 inhibition ratio	*200*	*1,600*	*150*	*50*
Pili per cell	*1.1*	*2.8*	*3.6*	*3.0*
% hybridisation to:				
λtraJ	*0.065*	-	*0.54*	-
λtraALEKBVWCU	*0.24*	-	*1.28*	-
λtraVWCUNFHGSTD	*0.35*	-	*1.25*	-
λtraIZ	*0.054*	-	*0.36*	-
Relative amount of:				
protein S (18kd) (OM)	*1*	*2.3*	*3.4*	*1.6*
protein T (25kd) (OM)	*1*	*3.8*	*3.0*	*2.8*
protein I (180kd) (CYT)	*1*	*4.5*	*4.7*	*3.4*

OM, CYT refer to outer membrane and cytoplasmic fractions.

to female-specific phage encoded by EcoRI fragment f7, and only pED805 carried the F *rep* and *inc* loci located on EcoRI fragment f5. The transfer-related properties of pED805, pED851 and pED862 are summarized and compared with those of F*lac* in Table I. As expected these multicopy plasmids expressed the *tra* functions to a greater extent than F*lac*. Their host cells were superior donors, poorer recipients (as measured by surface exclusion), had more pili per cell, produced more *tra*-specific protein; and for cells carrying pED851, more *tra*-specific mRNA from every part of the transfer region was detectable. What was unexpected were the relatively modest increases in protein and mRNA levels when compared to the very large increase in copy number. Compared to F*lac* a 40 fold increase in *tra* gene dosage for pED851 resulted in a 3.6-8.3 fold increase in mRNA level, a 3.4-4.7 fold increase in the amount of *tra* protein and a 3.6 fold increase in the number of pili per cell. The small values for the F-specific proteins could reflect deficiencies related to pilus assembly or to incorporation of some of these proteins into membranes resulting in degradation of the proteins. But when the values for the protein and mRNA levels were taken together the data suggested that a form of transcriptional control was being observed. The mechanism of this inhibition is unknown but is under investigation.

Cloning of these high molecular weight fragments demonstrated directly: the feasibility of such experiments; that the copy number was not dramatically lowered from that of pBR322; and that *E. coli* could stably accommodate 39% of its total DNA as plasmid when in logarithmic growth.

TRANSPOSITION OF THE F-TRANSFER GENES

Insertion of the 44.5Kb BamHI fragment of F into the unique BamHI site of the Tn*1* inserted into pDS1106 (ColE1::Tn*1*, see reference 3) resulted in the formation of a new transposon, Tn*2301*, which was 10 times larger than Tn*1* (D.A. Johnson and N.S. Willetts, in preparation). As determined by restriction analysis this recombinant has been tentatively assigned the structure shown in Figure 2.

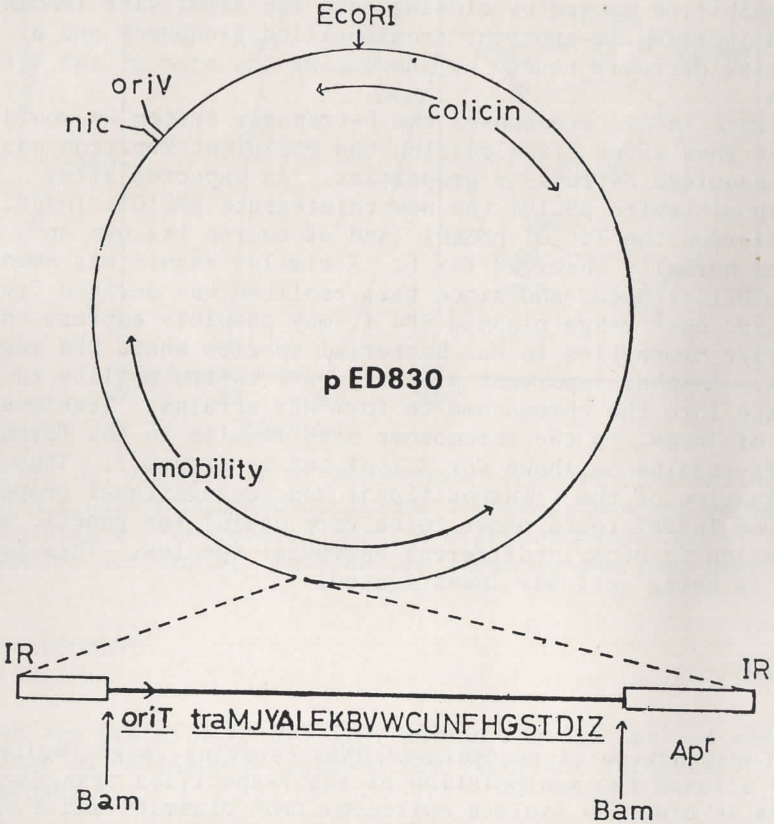

FIG. 2. Structure of pED830 containing the tra$^+$ *transposon. IR refers to the inverted repeats at the ends of* Tn1.

Bennett and Richmond (2) estimated the frequency of transposition of Tn*1* to vary between $1x10^{-2}$ and $2x10^{-6}$/cell for translocations from the chromosome to various R factors. Similar analysis for Tn*3* which is closely related if not identical to Tn*1*, gave a value of $7x10^{-4}$/cell (6). Using as an assay the mobilization of the small non-conjugative plasmids, pSC101 or pED712 (a Tc^{R} mini-RP4, J.M. Watson and N.S. Willetts, in preparation), we estimated the frequency of transposition of Tn*2301* to lie between $1.6x10^{-4}$ and $4.5x10^{-6}$/cell. These values agree well with those measured for Tn*1* and Tn*3* before and with the value of $5x10^{-4}$/cell measured for Tn*3* in our hands. Thus we conclude that a very large increase in the size of this transposon did not appreciably reduce its ability to translocate. Two research groups (4,5,6) have demonstrated that the region in Tn*1* (or Tn*3*) near the BamHI site is involved in inhibition of translocation. Our observed values may then be the result of two factors, the relaxation of inhibition caused by cloning into the BamHI site leading to an increase in apparent transposition frequency and a putative decrease caused by increasing the size of the transposon.

Since Tn*2301* transposed the F-transfer system we would expect that after translocation the recipient replicon would have acquired F-transfer properties. As expected after transposition to pSC101 the new cointegrate pSC101::Tn*2301* transferred the Tc^{R} of pSC101 (and of course its own Ap^{R}) at values normally observed for F. A similar result was seen for pED712::Tn*2301* and since this replicon was derived from the wide host range plasmid RP4 it may possibly express conjugative properties in the bacterial species where RP4 replicates. Another important property of F is its ability to integrate into the chromosome to form Hfr strains. Transposition of Tn*2301* to the chromosome also results in the formation of Hfr strains as shown for 3 isolates in Figure 3. Thus a combination of the transpositional and conjugational properties of Tn*2301* could prove to be very useful for genetic manipulation *in vivo* in different bacterial species. This prospect is being actively investigated.

SUMMARY

Construction of recombinant DNAs carrying large regions of F allowed the manipulation of the F-specified transfer genes in order to isolate multicopy *tra*$^{+}$ plasmids and a transposon which transposes the F-conjugation system.

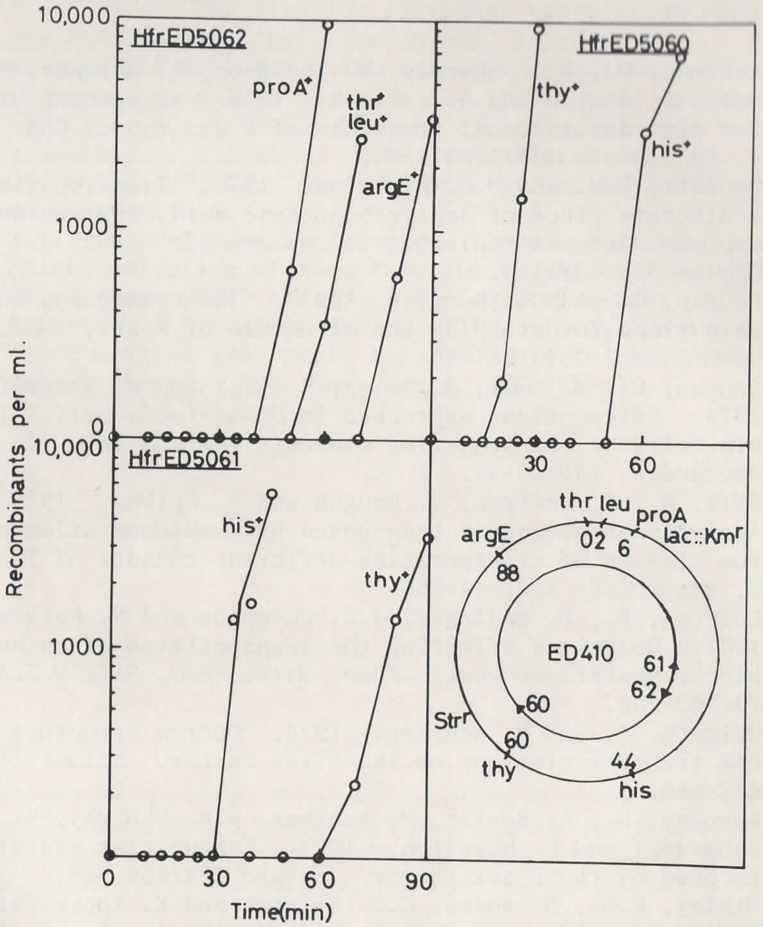

*Fig. 3. Appearance of chromosomal markers from matings of donor Hfr strains formed by Tn*2301 *transposition and recipient strain ED410. The deduced positions and orientations of the Hfr strain origins of transfer are shown in the last panel.*

ACKNOWLEDGMENTS

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PLASMID CURING DURING THE FORMATION AND REGENERATION OF PROTOPLASTS IN *STAPHYLOCOCCUS AUREUS*[1]

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It has long been considered likely that there is a functional association between DNA and the bacterial cell membrane involving replication or partition or both. The clearest formulation of this concept was by Jacob et al. (4) as part of their classic replicon model; this formulation has served to guide much of the subsequent investigation of plasmid autonomy functions. Thus Kawakami and Landman (5,6) attempted to test the hypothesis that plasmids were attached to the mesosome. This attempt was based on the finding by Ryter and Landman (13) that mesosomes were extruded and lost from the cell during protoplast formation; thus protoplasting should cure plasmids if the hypothesis were correct. For technical reasons, Kawakami and Landman were unable to obtain evidence either for or against the hypothesis; nor has any further investigation of this possibility been reported in the intervening years.

The question has recently been reopened by a finding of Sanchez-Rivas and Schaeffer (personal communication) that a *Bacillus subtilis* strain quantitatively lost the ability to produce a certain bacteriocin as a consequence of the formation and regeneration of protoplasts. As it was not clear from the available data whether or not a plasmid was involved in the determination of this bacteriocin, we decided to investigate the phenomenon with well-characterized plasmids in *S. aureus*.

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Table 1. Plasmid Curing in Regenerated Protoplasts

Strain	Plasmids: Designation	Phenotype	M.W. (Mdal)	Copies/cell	Curing frequencies[a]: Regenerants	Controls
RN1801	pT127	Tc^r Inc^3	2.7	50	40(159/535)	<0.2%(0/697)
RN1305	pC221	Cm^r Inc^4	3.0	20	28(107/387)	<0.25(0/472)
RN1777	pS177	Sm^r Inc^5	2.8	20	25(52/205)	
RN2902	pSH1	Tc^r	2.7		14(30/208)	
RN2425	pC194	Cm^r Inc8	1.8		13(102/782)	
RN2393	pUB110	Km^r	3.0		30(9/30)	<0.2(0/571)
BM3103	pIP687	Cd^r	2.6		21(21/100)	<0.2(0/561)
RN453	pI258	Pc^r Cd^r Hg^r	18.6	8-10	5.3(22/411)	<0.15(0/806)
RN1465	pII147	Asa^r Em^r Inc2 Pc^r Cd^r Hg^r	21.4	8-10	0.5(1/208)	<0.3(0/368)
RN29-3	pI6187	Asa^r Inc2 Pc^r Cd^r Hg^r	15.4	8-10	1(2/208)	
RN2466	pI258Δ94 (=pRN2467)	Asa^r Inc1 Em^r Inc1	7.0	8-10	<0.5(0/200)	
RN1962	pT127	Tc^r Inc3	2.7		79(199/253)	<2.0(0/697)
	pS177	Sm^r Inc5	2.8		49(124/253)	
	pC221	Cm^r Inc4	3.0		62(153/253)	
	pK545	Km^r Inc6	14.6	2-3	3(7/253)	
	pI258	Pc^r etc Inc1	18.6	1-2	0.8(2/253)	<0.2(0/787)

[a] Curing frequencies are given as percent cured among total scored, the actual figures are given in parentheses.

In these experiments, exponentially growing organisms in CY broth (9) were pelleted by centrifugation, resuspended in sucrose-maleate-magnesium chloride (SMM) buffer (14) and treated with lysostaphin (15 μg/ml). After protoplast formation was complete as revealed by monitoring for lysis with 1% sarkosyl, the protoplasts were washed twice with SMM by centrifugation and then diluted and plated on a regeneration medium (15) and on a standard medium to score for survival of non-protoplasted cells. In these experiments, survival on the standard (non-protective) medium was generally less than 10^{-8} and the regeneration frequency was usually about 10%. Regenerants were scored by testing for the plasmid-associated resistance phenotypes; a set of selected regenerants was also subjected to lysis and agarose gel electrophoresis according to the method of Eckhardt (1).

The intial results were that plasmids were indeed lost as a consequence of protoplast formation and regeneration of whole cells. Typical results for a variety of *S. aureus* plasmids are listed in Table 1. Curing frequencies ranged from 13% to 80% of regenerants for members of a group of nine small (1.8-3 Mdal) plasmids and these frequencies were reproducible. A second group of plasmids showed curing frequencies that were only slightly, if at all greater than their spontaneous loss frequencies. The latter are all larger (15-21 Mdal) and present in fewer copies than the former. For the small plasmids, the presence of other plasmids in the cell appeared to exert a significant effect on curing frequencies. Strain RN1962 carries five plasmids (12), three curable and two non-curable; the former were cured at frequencies that were considerably greater when they were in the multiplasmid strain than when alone in the same host background. The non-curable plasmids were not cured at a significant frequency in either case.

That phenotypic curing was associated with the loss of plasmid DNA, was demonstrated by agarose gel electrophoresis of whole cell lysates. In Figure 1 is shown a representative gel electrophoresis pattern for a series of regenerants of RN1962 that have lost various combinations of the plasmid-associated phenotypes.

The possibility that curing was related to molecular weight was made less likely by the observation that pI258Δ94, a derivative of pI258 with a deletion of 2/3 of its genome and a resultant molecular weight of about 7 Mdal (11) was cured even less frequently than its parent plasmid. As the small (1.8-3 Mdal) plasmids all had copy numbers greater than the large ones, it would appear that curing was not a function of low copy number either.

An attempt to determine where curing occurred in the protoplast formation and regeneration cycle involved the following

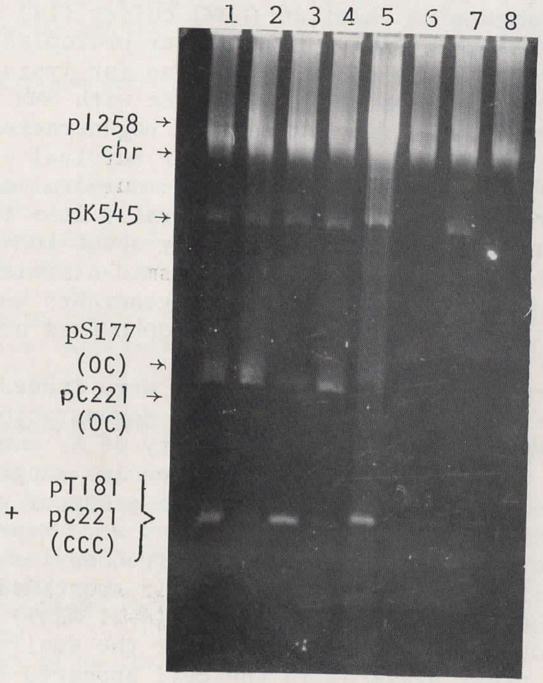

Figure 1. Correlation of plasmid content of RN1962 regenerants with phenotype. Regenerant colonies were scored for resistance phenotype, then analyzed for plasmid DNA content by the agarose gel method of Eckhardt (1). Lane 1: RN1962; lane 2: Tc^s *regenerant; lane 3:* Cm^s, Sm^s*; lane 4:* Tc^s, Sm^s*; lane 5:* Cm^s*; lane 6:* Tc^s, Sm^s, Cm^s, Km^s*; lane 7:* Tc^s, Sm^s, Cm^s*; lane 8: RN450 (no plasmid). The arrows indicate locations of various DNA species: pI258 = pI258 CCC DNA; pK545 = pK545 CCC DNA; C = open circular DNA; CCC = supercoiled DNA. Note that the sodium dodecyl sulfate (SDS) used in the lysis procedure converts the relaxation complexes of pS177 and pC221 (8) to the open circular configuration.*

series of experiments: (i) to test the possibility that plasmolysis, *per se*, was responsible, we scored several cultures for curing after resuspension in SMM but without lysostaphin treatment. The results, listed in Table 1 under "controls", were that no curing could be detected under these conditions. (ii) We were unable to detect any plasmid DNA in supernatants of protoplasts after centrifugation; moreover, the ratio of plasmid to chromosomal DNA was the same in protoplasts as in whole cells (Figure 2, lanes 1-3 and Table 2). (iii) On the basis of these two experiments, we concluded that curing most probably occurred during the regeneration of the protoplasts. If so, it must be a consequence of abnormal replication or of abnormal partition during regeneration. A test of the former

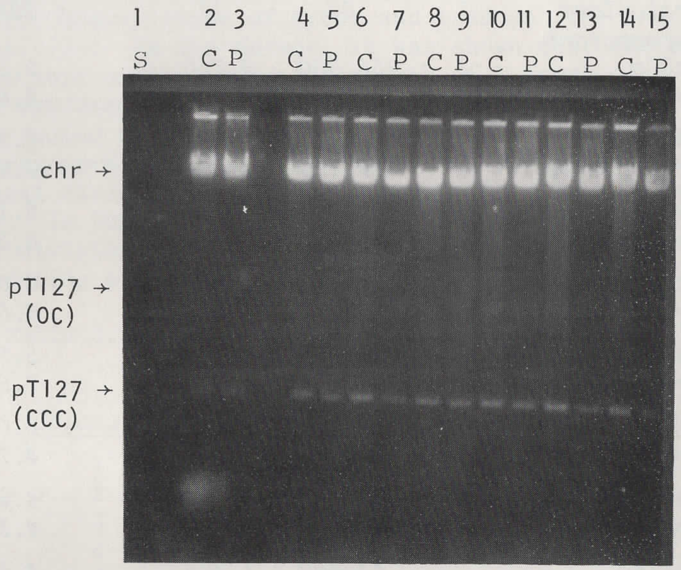

Figure 2. Effect of protoplast formation on plasmid content and replication. Protoplasts of RN1801, labeled with ^3H *thymidine, were centrifuged, resuspended in SMM, lysed with SDS, sheared and analyzed by gel electrophoresis (lane 3) in comparison with a whole cell lysate (lane 2) and with a protoplast supernatant (lane 1). DNA-containing regions of the gel were excised and counted (see Table 4). Suspensions of unlabeled protoplasts and of whole cells in SMM with .5% casamino acids and .5% yeast extract (Difco) were then incubated for 180 min. At 30 minute intervals, aliquots were removed, incubated for 10 min. with* ^3H *thymidine (10* $\mu Ci/ml$*) and then subjected to lysis and analysis on agarose (channels 4-15). In this gel, the lowest band is pT127 CCC monomers, the next is OC monomers and the heavy upper band is chromosomal DNA.*

Table 2. Recovery and Replication of Plasmid DNA in Protoplasts

			^3H Counts recovered		Incorporation of ^3H-Thy in plasmid
Time	*Sample*	*Lane (Fig. 2)*	*Total*	*Plasmid DNA band*	*(% of total)*
Prelabel	protoplast supernatant	1	61	11	<.001[a]
	cells	2	150,000	3,750	2.5
	protoplasts	3	165,000	4,540	2.8
0-10	cells	4	9,700	704	7.2
	protoplasts	5	16,100	703	4.4
30-40	cells	6	10,700	659	6.1
	protoplasts	7	8,860	512	5.8
60-70	cells	8	12,200	832	6.8
	protoplasts	9	19,800	1,310	6.6
90-100	cells	10	17,700	920	5.2
	protoplasts	11	28,300	2,140	7.6
120-130	cells	12	30,000	1,510	5.0
	protoplasts	13	38,400	1,820	4.7
150-160	cells	14	37,900	1,710	4.5
	protoplasts	15	55,700	2,370	4.3
180-190	cells		43,400	1,960	4.5
	protoplasts		98,700	3,270	3.3

[a] *Less than 0.01% of the total counts were recovered in the protoplast supernatant.*

possibility was negative: in an experiment in which the incorporation of ^3H-labeled thymidine was monitored in whole cells and protoplasts during three hours of incubation in SMM plus a nutritional supplement consisting of 0.5% casamino acids plus 0.5% yeast extract, there was no apparent difference between the two cell types (results listed in Table 2 and shown in Figure 2, lanes 4-15). (iv) Finally, we considered the possibility that curing occurred as a consequence of abnormal partition-division during regeneration. Direct evidence for this possibility was not obtained: however, an indirect test seemed to support it: regenerant colonies of RN1801 (carrying pT127) were picked in their entirety, emulsified in buffer, then diluted and plated for plasmid positives and negatives. The result of this test, shown in Table 3, was a striking distribution of plasmid positive and negative organisms within the regenerant colonies. In the small sample of 16 colonies, the entire range of cell population distributions was observed, and there were only three that had no detectable negatives. Thus, if one considers mixed colonies as in some sense cured, then the curing frequency for the plasmid studied (pT127) was about 80% of the regenerants.

In considering these results, we refer to two previous papers, namely those of Ryter and Landman (13) and of Hadlaczky et al. (2). In the former, it was shown that the cell divisions that occur during the growth of L forms of *B. subtilis* were extremely abnormal; cells of all sizes were produced and many lacked nucleoids. In the latter, it was shown that during regeneration, *Bacillus megaterium* protoplast underwent several divisions before any detectable cell wall was laid down. In interpreting our results we assume that these pre-wall divisions are analogous to L form divisions and that regenerating protoplasts of *S. aureus* behave similarly to those

Table 3. Analysis of Regenerant Colonies of RN1801 (Carrying pT127)

Colony #	*Tc^r/total[a]*	*% Tc^r*
1	$0/10^7$	10^{-5}
2	$0/10^7$	10^{-5}
3	$0/10^7$	10^{-5}
4	$0/10^7$	10^{-5}
5	$0/10^7$	10^{-5}
6	$6 x 10^{-6}$	$6 x 10^{-4}$
7	$1.5 x 10^{-5}$	$1.5 x 10^{-3}$
8	4/200	2
9	76/1006	2.6
10	13/200	6.5
11	5/10	50
12	697/768	91
13	199/200	99.5
14	300/300	99.7
15	1072/1072	99.9
16	934/934	99.9

[a] *Single regenerant colonies were resuspended in their entirety, emulsified and scored by plating for the fraction of Tc^r cells. Except for colonies 1-5, which were re-plated in their entirety on Tc plates, all of the figures given represent the results of replica plating from nonselective to Tc plates.*

of the bacilli. Our interpretation, then, is simply that during the pre-wall divisions, there is frequent segregation of the plasmids from the nucleoid so that the former often end up in chromosome-less vesicles while the viable (i.e., chromosome-containing) cells often end up without plasmids. This interpretation implies that there is effective sequestration of the curable plasmids in a specific compartment of the cell that is separate from the nucleoid and that plasmid molecules are not free to diffuse at random throughout the cytoplasm of the cell. This is particularly striking with respect to pT127, the plasmid that has been studied most thoroughly in this connection. Since this plasmid has an average of 50 copies per cell (which corresponds to about 70 copies in cells about to divide), it is difficult to imagine that plasmidless cells would be generated with the high frequency observed if there were free diffusion. This conclusion is supported further by recent results obtained with a mutant of a very closely related plasmid, pT181 (3), that has an elevated copy number (about 250 per average cell). This mutant plasmid showed curing frequencies in regenerants that were in the range of 40%, which is about the same as those of the parent plasmid from which it was derived (S. Gruss and S. Carleton, personal communication). In view of previous suggestive evidence that these small tetracycline plasmids undergo equipartition (10), our present working hypothesis is that curing is a consequence of the profound disruption of the division-partition system that occurs during the abnormal divisions of the protoplasts prior to the regeneration of the cell wall, and that therefore the partition of these plasmids directly involves the cell envelope.

Considering those plasmids that are *not* cured as a consequence of protoplast regeneration, it would appear that they must be partitioned by association with some structure that is obligatorily associated with the chromosome, or perhaps the chromosome itself as suggested by Kline and Miller (7).

Further work in this area will involve attempts to demonstrate sequestration of plasmids during protoplast divisions and an analysis of the gene(s) responsible for the difference between the two classes of plasmids.

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DISCUSSION

Macrina: Did I understand you to say that you let the protoplasts sit for 3 hours before plating on regeneration medium?

NOVICK: No, that was simply to study replication of plasmids; we ordinarily plate them right away.

Macrina: At physiologic temperatures?

NOVICK: Yes. They're perfectly happy. People who work with *E. coli* and other Gram negatives are not used to protoplasts because they're very difficult to work with. But in Gram positives they're very easy to work with as long as you keep detergents out of your glassware. You can leave them overnight on the desk and they're fine in the morning.

ColE1 PLASMID REPLICATION CONTROL[1]

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CONTROL OF ColE1 DNA REPLICATION

Perhaps the most striking feature of extrachromosomal elements is their genetic stability. Despite their low genetic complexity, the average number of plasmid copies per cell is kept constant in an exponentially growing bacterial population. These two facts, high stability and low complexity, mean that plasmid DNA replication is carefully controlled and that plasmids represent important model systems for investigating how replicons decide to replicate.

We have been concerned with the question of how the colicinogenic plasmid ColE1 and its derivatives decide to replicate. ColE1 is a small replicon (molecular weight 4.6 x 10^6) which replicates under "relaxed" control, i.e., it exists normally at 10-15 copies per host chromosome and its replication proceeds extensively in the absence of host protein synthesis (1). In addition, ColE1 DNA replication requires DNA polymerase I for replication (7). ColE1 replicates unidirectionally from a unique origin located about 17% of unit length from the single *Eco*RI site (8).

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From the point of view of replication control it is essential to determine whether any plasmid encoded information directly affects the rate at which replication is initiated. With respect to ColE1 replication, it seems clear that no ColE1 encoded polypeptide is *positively* required for replication since replication proceeds extensively when protein synthesis is inhibited (26). To investigate whether other controls might be involved in vegetatively replicating ColE1, we have isolated and characterized copy number mutants of ColE1 derivatives.

Our experiments involved the cloning vehicle pBGP120 (10). pBGP120 was originally constructed to permit controlled genetic expression of heterologous DNA by placing it under the control of the *Escherichia coli lac* operator and promoter (10). Briefly, pBGP120 was constructed by ligation of a ColE1 derivative RSF2124 containing the Tn3 transposon (12) to a λ*plac*5 *Eco*RI DNA fragment carrying the *lac* operator, promoter, and most of the β-galactosidase structural gene (Z gene). One of the two *Eco*RI sites in the resulting plasmid was then deleted *in vitro*. The remaining EcoRI site in pBGP120 is located near the -COOH terminus of the Z gene. As a result, cells carrying pBGP120 synthesize a truncated β-galactosidase monomer which has enzymatic activity in a strain which can provide the -COOH terminal portion of the monomer via omega complementation (13). The structure of pBGP120 is shown schematically in Figure 1.

Our strategy for isolating copy number mutants of pBGP120 exploited the observation that, owing to its high copy number, pBGP120 titrates the *lac* repressor present in i^+ strains. Consequently, such strains exhibit partial constitutive synthesis of the truncated β-galactosidase monomer (9). However, transformation of pBGP120 into *E. coli* strain M96, which overproduces the *lac* repressor 50-fold (4), results in a repression of plasmid-mediated β-galactosidase synthesis (3).

Using indicator plates containing 5-bromo-4 chloro-3indolyl-β-D-galactoside (X-Gal) we observed that about 1.0% of M96 colonies carrying pBGP120 were constitutive for β-galactosidase production (3). When the plasmid content present in cleared lysates from these clones was examined large amounts of a supercoiled DNA species identical in molecular size to pBGP120 were observed (Figure 2). The plasmid in one such colony was designated pOP1. Comparison of restriction endonuclease fragment patterns between pOP1 and pBGP120 indicated that pOP1 was similar in nucleotide sequence arrangement to pBGP120.

To demonstrate that the mutation responsible for DNA overproduction in pOP1 occurred in the plasmid genome, we transformed a variety of *E. coli* strains with purified pOP1 DNA.

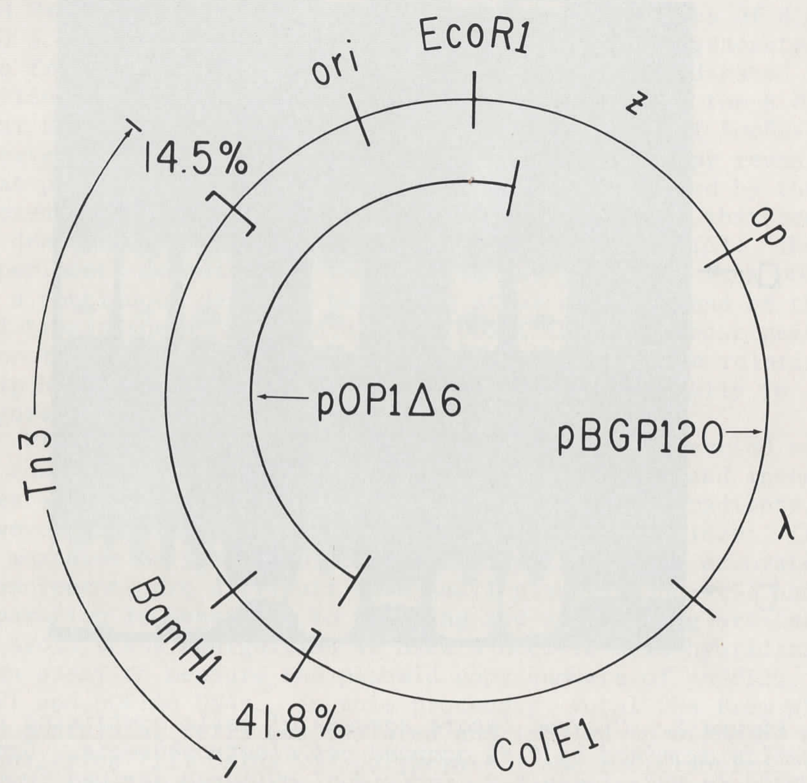

Figure 1. Schematic diagram of pBGP120 and pOP1Δ6 DNAs. Ori, ColE1 origin of replication; op, operator and promoter of the lac *operon; Z, structural gene for β-galactosidase. Percentages refer to counterclockwise measurement from the* Eco*RI site. Inner line, sequences retained after the deletion which generated pOP1Δ6 from pOP1.*

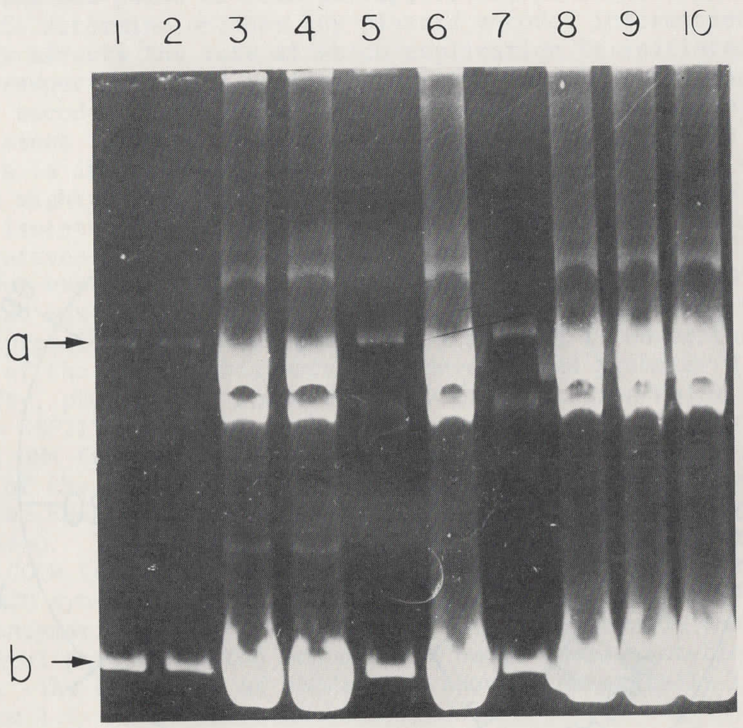

Figure 2. Cleared lysate screens of cells containing plasmids examined by 0.8% agarose gel electrophoresis. Lane 1, pBGP120 in M96; lanes 2-7, individual cultures derived from six blue M96 colonies after transformation with pBGP120; lanes 8-10, cleared lysates from three blue, Ap^r colonies arising after transformation of strain M96 with DNA contained in the cleared lysate shown in lane 3. Arrows marked a *and* b *indicate positions of chromosomal and supercoiled pBGP120 DNA respectively.*

pOP1 exhibited an overproducer phenotype relative to pBGP120 in strains M96, DG74 (Cya$^-$), and HB101 (Rec$^-$). Taken together with the restriction endonuclease digestion data, this result indicated that pOP1 probably contained a point mutation in a region of plasmid DNA involved in copy number control.

In certain strains we observed that pOP1 was structurally unstable. After transformation of *E. coli* strain DG75, we were unable to detect pOP1-sized plasmids. Instead we observed a 4.6 x 10^6 molecular weight plasmid which retained the DNA overproducer phenotype of pBGP120. The 4.6 megadalton (Mdal) plasmid, designated pOP1Δ6, was compared to pBGP120 by restriction endonuclease fragment analysis and by heteroduplex analysis. Double digestion of pBGP120 or pOP1 DNA with EcoRI and *Bam*H1 generated two fragments of molecular sizes of 8.0 and 3.5 Mdal. Similar double digestion of pOP1Δ6 generated two fragments of 3.6 and 1.1 Mdal. This result indicated that pOP1Δ6 was derived by a deletion which occurred in the 8.0 Mdal *Eco*RI-*Bam*H1 fragment of pOP1 DNA. Analysis of *Eco*RI-cleaved, denatured pOP1Δ6 DNA by electron microscopy revealed that pOP1Δ6 retained the Tn3 transposon as indicated by the presence of a stem and loop structure identical to that seen in denatured pBGP120 DNA and characteristic of Tn3(5). These experiments demonstrated that pOP1Δ6 was generated from pOP1 by a continuous deletion beginning at or near one end of the Tn3 transposon and extending to a point about 600 base pairs from the *Eco*RI site in the Z gene of pOP1 DNA. The relationship between pOP1Δ6 and pBGP120 is shown schematically in Figure 1.

Plasmid copy number determinations are often carried out by comparison of chromosomal and supercoiled DNA band intensities after equilibrium sedimentation in CsCl-dye gradients. However, this procedure has inherent limitations since: (1) it measures only supercoiled plasmid species; (2) accurate measurements are difficult with small plasmids and (3) band separation is sensitive to trapping and overloading artifacts. To avoid these ambiguities we used a nucleic acid hybridization assay to measure the plasmid copy numbers of pBGP120, pOP1 and pOP1Δ6 DNAs. In this procedure, total DNA from plasmid containing cells was isolated and labeled *in vitro* by nick translation (11) using DNA polymerase I and radiolabeled nucleoside triphosphates. Copy number determination was made by driving the resulting single-stranded labeled tracer into hybrid form with excess purified plasmid DNA. Because of the low sequence complexity of plasmid DNA the portion of the labeled tracer complementary to plasmid DNA was rapidly driven into hybrid form. The hybridization reaction was monitored by chromatography on hydroxyapatite. The fraction of tracer bound to hydroxyapatite at the end of the reaction was equal to the fraction of total cell DNA that was plasmid specific. Knowing the molecular size of the plasmid it was a simple matter to calculate copy number from these data.

Copy number data for pBGP120, pOP1 and pOP1Δ6 were obtained using the saturation hybridization procedure (Table 1). The data indicated that 6% of the DG75/pBGP120 tracer was driven into hybrid form by excess pBGP120 DNA. In contrast, 30% of tracer from either pOP1 or pOP1Δ6 containing cells was driven into hybrid by pBGP120 DNA. These data correspond to copy numbers of 15, 74 and 210 per host chromosome for pBGP120, pOP1 and pOP1Δ6 respectively. When tracers were prepared from

Table 1. Plasmid Copy Numbers

Host/plasmid	*% intracellular DNA* -CAM	*% intracellular DNA* +CAM[b]	*Plasmid copy number*[a] -CAM	*Plasmid copy number*[a] +CAM[b]
DG75/pBGP120	6	45	15	185
DG75/pOP1Δ6	27	36	210	300
DG75/pNOP1	3	40	15	350
DG75/pBGP120+pOP1Δ6	1.5	16	2[c]/5[d]	20[c]/60[d]

[a] *Plasmid copy number was calculated from the weight ratio of plasmid and chromosomal DNA. The molecular weight of the DG75 chromosome is 3.75 x 10^6 bp. The percent intracellular DNA as plasmid is the average of three plateau values for each determination. Copy number is expressed per chromosome mass equivalent.*

[b] *Cultures were treated for 17-18 hours with 250 µg/ml chloramphenicol (CAM). Relative copy numbers were estimated by integrating scans of gels of cleared lysate DNA stained with ethidium bromide.*

[c] *pBGP120*

[d] *pOP1Δ6*

chloramphenicol treated, plasmid-containing cells, the copy numbers per host chromosome were 185, 114 and 300 for pBGP120, pOP1 and pOP1Δ6 respectively.

We investigated the levels of expression of two plasmid encoded polypeptides, β-galactosidase and β-lactamase. The plasmid copy number (Cop^-) mutants showed increased levels of expression of these genes. More than 50% of the total cellular protein synthetic capacity is directed to β-galactosidase production in cells carrying pOP1 after induction with cyclic AMP (3). Similarly, the specific activity of β-lactamase in crude extracts of cells carrying pOP1Δ6 was about 12-fold greater than that observed in extracts of cells carrying pBGP120. This increase corresponded closely to the difference in copy number between pOP1Δ6 and pBGP120 and was consistent with observations of Nordström and co-workers that plasmid encoded β-lactamase production is gene-dosage dependent.

To investigate the nature of the replication control function altered in the copy number (Cop^-) mutants pOP1 and pOP1Δ6, we introduced both pOP1Δ6 and pBGP120 into strain DG75 simultaneously by co-transformation. The transformation was done with a ten-fold molar excess of pOP1Δ6 DNA (Ap^rLac^-) relative to pBGP120 DNA (Ap^rLac^+). Transformants were selected on minimal plates containing lactose and ampicillin (Ap). About 20% of the Ap^rLac^+ transformants harbored both plasmids. When cleared lysates were prepared from co-transformants and plasmid DNA examined by agarose gel electrophoresis, both pBGP120 and pOP1Δ6 appeared to be present in low copy number (Figure 3, lane 1) relative to pOP1Δ6 when present alone in the same host (Figure 3, lane 3). One clone with co-resident plasmids, designated PS001, was studied further. When the copy numbers of pOP1Δ6 and pBGP120 were measured in PS001 by the saturation hybridization assay they were found to be 2 and 5 respectively (Table 1).

These results indicated that the Cop^- phenotype of pOP1Δ6 was suppressed when it was co-resident with the wild type parent replicon pBGP120.

It is noteworthy that both plasmids are very stable as co-residents despite their high degree of relatedness. After 100 generations of growth in non-selective medium the segregation frequency of pBGP120 from pOP1Δ6 was less than 2 x 10^{-4} per cell. These results indicated a high degree of stability in the association and suggested that pOP1Δ6 may be altered in incompatibility properties as well as replication control.

To assess the specificity of the depression of pOP1Δ6 copy number in *trans* by pBGP120 we asked whether an unrelated plasmid, pSC101, could affect pOP1Δ6 copy number. Accordingly, strain DG75 was co-transformed with pOP1Δ6 and pSC101 DNAs and Ap^rTc^r transformants were selected. pSC101 confers resistance to tetracycline (Tc). Figure 3, lane 8 shows an agarose gel analysis of a cleared lysate from such a double transformant. It is apparent that pSC101 had no detectable effect on pOP1Δ6 copy number.

These results indicated that pOP1Δ6 carried a mutation in a replicon-specific, *negatively* acting function involved in replication control and that the mutation could be complemented by the wild type gene product *in trans*.

We used recombinant techniques *in vitro* to map the mutation responsible for DNA overproduction in pOP1Δ6. The basic strategy of these experiments was to prepare cognate restriction endonuclease fragments from wild type and overproducer plasmids, link them to a non-replicating fragment carrying a selectable marker, introduce the recombinants into a recipient by transformation, and screen the transformants qualitatively for plasmid DNA content. With this approach we first showed that the *cop* mutation in pOP1Δ6 was located on the 3.5 Mdal fragment containing the plasmid origin of replication. Using the 3.5 Mdal *Eco*RI-*Bam*H1 fragment from pBGP120 linked to a non-replicating 1.1 Mdal fragment of pOP1Δ6 carrying the β-lactamase gene we generated a Cop^+ plasmid identical in molecular size to pOP1Δ6. This Cop^+ plasmid was designated pNOP1 (NOP signifies non-overproducer). pNOP1 existed intracellularly

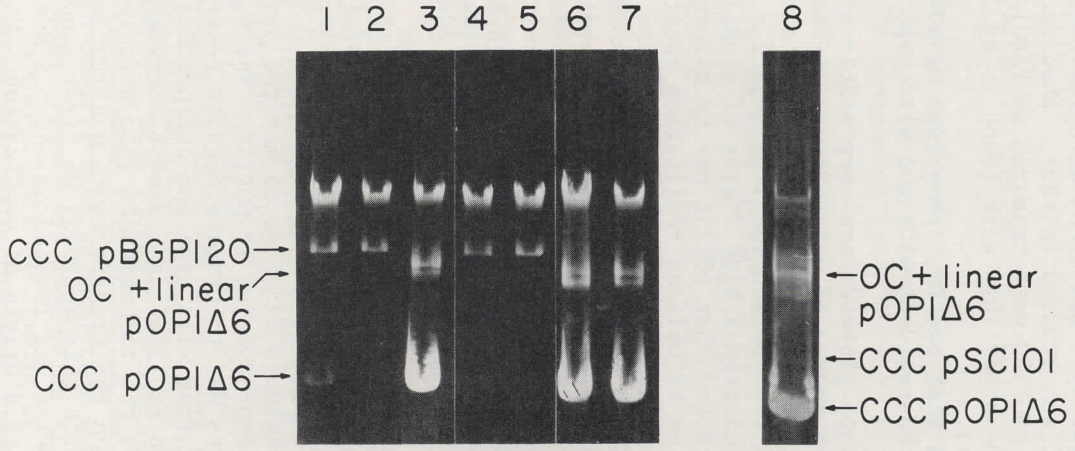

Figure 3. The effect of pBGP120 and pSC101 co-residents on pOP1Δ6 copy number. 0.8% agarose gel analysis of plasmid DNA in cleared lysates of DG75/pOP1Δ6 + pBGP120 (PS001; lane 1), DG75/pBGP120 (lane 2), and DG75/pOP1Δ6 (lane 3). Also shown is a similar analysis of plasmid DNA content of Ap^{r}Lac$^+$ transformants (lanes 4 and 5) and Ap^{r}Lac$^-$ transformants (lanes 6 and 7) obtained after transformation with plasmid DNA from the DG75/pOP1Δ6 + pBGP120 co-resident as described in the text. Lane 8 shows DNA in a cleared lysate from DG75/pOP1Δ6 + p SC101. "CCC" represents covalently closed circular DNA, and "OC" is open circular DNA. The upper band in each lane is chromosomal DNA.

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at 15 copies per chromosome (Table 1), an identical copy number to that shown by its parent, pBGP120. It is noteworthy that ColE1 also exists at 15 copies per chromosome. Thus, despite the 2.5 fold difference in molecular size between ColE1, pNOP1 and pBGP120, each of these replicons exists at the same copy number.

From the copy number data shown in Table 1, it is apparent that replication of the overproducer mutants pOP1 and pBGP120 was not controlled by the same parameters as the Cop$^+$ parent plasmids. Despite the 2.5 fold difference in size between pOP1Δ6 and pOP1 DNA, both plasmids comprised the same percentage (about 30%) of total intracellular DNA.

These observations suggest that some critical plasmid encoded control element normally functions by sensing the number of plasmid copies, perhaps the origin concentration (11), and acts negatively to repress replication. What is sensed is independent of molecular size. However, when the repressor is inactivated by mutation, origin concentration is no longer sensed and replication is released from repression and becomes limited by other factors, e.g., host replication machinery. Thus, copy number and molecular size become related, perhaps to the extent that the cell can tolerate extra DNA.

The fact that pOP1Δ6 responded to the pBGP120-encoded repressor suggests that pOP1Δ6 retains a wild type repressor binding site, and implies the existence of dominant overproducer mutations in these ColE1 derivatives which would be insensitive to repressor action *in trans*.

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DISCUSSION

Shapiro: Is there any evidence to show that the repressor is not a membrane protein?

POLISKY: There's no evidence to show that, although that's the view I've also held. I would like to suggest that it may not be coincidence or randomness that the immunity gene maps right next to the origin. The overproducer is colicin E1-immune.

So, if you adhere to the immunity possibility, you have to say it's a multifunctional protein. There are some attractions in the membrane protein idea, because, for example, it could provide a site on the membrane which could be negatively acting. I think the answer will come from the sequencing. I should also say that the over-producer mutant we have isolated is a point mutation; it reverts, so it's reasonably well behaved.

Novick: I'm not sure if you could do this control, but would a wild-type counterpart show incompatibility?

POLISKY: We've been talking about doing that experiment, and that's why I don't want to say anything about incompatibility until we do it. I don't want to give you the impression we know what the compatibility-incompatibility situation is. All I want to do here is to say that phenomenologically we know that the plasmids reside stably.

Falkow: My impression was that it was possible to isolate COL^{imm} mutants that do not have altered plasmid copy numbers.

POLISKY: That's basically true. However, the copy number determinations in this case have not been done very precisely.

Falkow: If you put them on an agarose gel and they look like yours, I doubt that many people would disagree.

POLISKY: That's true, and that's another reason why the immunity idea has little popularity.

Clewell: What about chloramphenicol amplification?

POLISKY: We measured that by the same experimental procedure described earlier. These plasmids are slightly amplifiable essentially up to the wild type level. So in a sense they offer no possibility for making more DNA. If you're interested in making DNA, you should simply amplify with chloramphenicol. But if you're interested in expression, these plasmids will give you an increased gene dosage.

Clewell: Would you say that the basis for amplification might be the inhibition of that protein?

POLISKY: That's one possibility but certainly not the whole story. Chloramphenicol amplification is probably caused by at least two things: inhibition of synthesis of the repressor; and because host chromosome replication is blocked, a lot of positive factors are released that are now available for a ColE1 replication.

CHARACTERIZATION OF AN H2 GROUP PLASMID MUTANT DEFECTIVE IN MAINTENANCE AND TRANSFER, AND ELIMINATED BY NOVOBIOCIN[1]

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We have isolated a temperature-sensitive mutant of an incompatibility group H2 plasmid which is also eliminated by treatment with novobiocin at 30°C. The wild-type plasmid pSD114 mediates resistance to chloramphenicol, kanamycin, streptomycin, tetracycline, potassium tellurite and to various coliphages (Phi). It originated from a strain of *Salmonella anatum* isolated in Ontario, Canada in 1974 (15). It is important to understand how H plasmids are replicated and maintained in bacterial cells because the loss of these plasmids in culture from strains of *Salmonella* has posed a problem for the antibiotic resistance in this genus (3,14,16). As part of our study of the maintenance of H group plasmids, we have isolated a temperature-sensitive mutant of the H2 plasmid pSD114. The mutant designated pDT4 was isolated by a method used for the selection of Phi$^-$ mutants, that is plasmids that do not mediate phage inhibition at 37°C. Cells of *Escherichia coli* RG176 (13) harboring the plasmid pSD114 were mutagenized with ethyl methane sulfonate (EMS) using the method of Miller (7), and were plated on LBC agar (9), which had been spread with phage T5 at a concentration of 10^6 pfu per plate. Clones of cells that allowed the development of phage T5 appeared as "nibbled" colonies. Colonies were picked and checked for the ability of the mutant plasmid to inhibit phage development in RG176 and after transfer to *E. coli* RG488 (13). Some of the clones obtained by this method were not Phi$^-$. In the case of pDT4 the mutation was in a maintenance function(s) rather than in the

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phage inhibition function. To study the elimination of the pDT4 plasmid, cultures of *E. coli* RG192 (pDT4) and RG192 (pSD114) were incubated for 18 hrs at 30°C in 1 ml of Penassay broth (Difco antibiotic medium No. 3) without shaking; each culture was diluted into 1 ml of fresh, prewarmed broth at 30, 35, 37, 42 or 44°C to give a final concentration of about 5,000 cells/ml. The cultures were grown for 24 hours without shaking, then plated for single colonies on MacConkey agar (Difco). The colonies were tested for the presence of the plasmid by replica-plating on MacConkey agar containing chloramphenicol (8 μg/ml), kanamycin (8 μg/ml), streptomycin (10 μg/ml), tetracycline (8 μg/ml); and Brain Heart Infusion agar (Difco) containing potassium tellurite ($2x10^{-5}$ M). Elimination of plasmid DNA was monitored by extraction of DNA and electrophoresis on agarose gels by the method of Hansen and Olsen (4). Loss of all drug resistance markers was accompanied by loss of the $166x10^6$ dalton plasmid pDT4 when visualized on agarose gels, as described previously (15). After overnight culture (20 generations of growth) in Penassay broth at 30°C, the pDT4 plasmid was maintained in host cells. After culture at 35°C, less than 1% of clones had lost the plasmid, however, after 20 generations of growth at 37, 42 or 44°C, up to 100% of clones had lost the plasmid. In contrast, pSD114 was maintained at all temperatures from 30° to 44°C.

The kinetics of loss of pDT4 at 37°C are shown in Figure 1. Cultures of RG192 containing pDT4 or pSD114 were grown at 30°C for 18 h in Penassay broth, then diluted into 30 ml of Penassay broth at 37°C to give a final concentration of about 10^3 cells/ml. Elimination of pDT4 was monitored by replica-plating on antibiotic medium. The results are shown as solid lines in Figure 1. The presence of pDT4 in cells of *E. coli* appeared to interfere with host cell growth, by extending the lag phase (dashed lines). Cultures of RG192 (pDT4) required 6 h of incubation before the same mean doubling time was achieved. By this time, most of the cells which originally contained pDT4 were plasmid-free (Figure 1).

Not only is pDT4 lost from host cells at high temperature, it can also be eliminated from cells at 30°C by treatment with novobiocin. The antibiotic is known to inhibit DNA replication by its interaction with bacterial DNA gyrase (2). DNA gyrase catalyses the supercoiling of circular double-stranded DNA in *E. coli* and is composed of two subunits. Subunit A is inhibited by nalidixic acid (5) whereas subunit B is inhibited by novobiocin or coumermycin A_1 (2). Novobiocin and coumermycin block ATP binding to DNA gyrase (8,12). DNA gyrase is required for the replication of the small plasmids RSF1030 and ColE1 (2,11). Some plasmids are eliminated by novobiocin treatment whereas others are not (6). These facts suggested that certain plasmids may code for their own DNA gyrase which is insensitive to novobiocin.

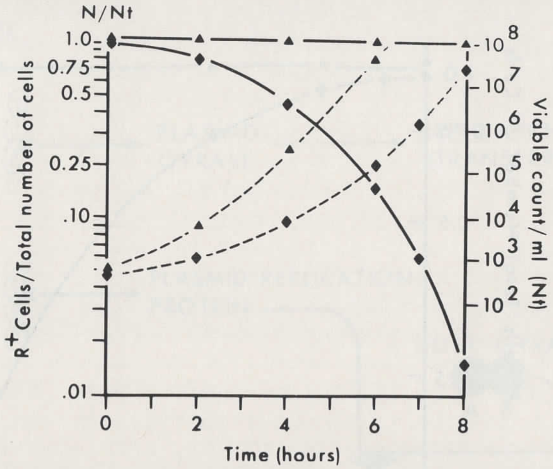

Figure 1. Elimination of pDT4 plasmid at 37°C. The R^+ derivatives of E. coli *K12 RG192 containing pSD114 (▲) or pDT4 (◆) were grown for 18 h at 30°C, diluted as described in the text, then incubated at 37°C. Samples were plated periodically to determine the total cell count (Nt, shown as dashed lines) and the number of cells that contained plasmids (N, shown as solid lines).*

Treatment with novobiocin eliminated pDT4 from its *E. coli* host at 30°C. As the concentration of novobiocin was increased, the percentage of cured cells also increased, for concentrations of 10 to 100 μg/ml (Figure 2). Novobiocin at 100 μg/ml reduced the efficiency of plating (EOP) of RG192 (pDT4) by 3 to 4 fold. Whereas at 150 μg/ml, the EOP was reduced about 10 fold and a lower frequency of curing was obtained (about 20%). Novobiocin treatment at 30°C reduced the EOP of R^+ and R^- strains by the same amount.

Coumermycin A_1 is known to inhibit DNA replication by the same mechanism as novobiocin, and killing is more rapid. Growth of overnight cultures with coumermycin at 30°C in the range of 10 to 12 μg/ml gave about 40% of clones which had lost the plasmid. Other curing agents were also tested. Neither ethidium bromide at concentrations of 1 to 20 μg/ml nor acridine orange at 10 to 100 μg/ml could eliminate pDT4 at 30°C. Nalidixic acid was also tested because it inhibits DNA replication by its interaction with the A subunit of DNA gyrase (5). Growth of RG192 (pDT4) cultures with 1 to 5 μg/ml nalidixic acid did not result in curing. Therefore pDT4 seems to be eliminated only by agents which interact with the B

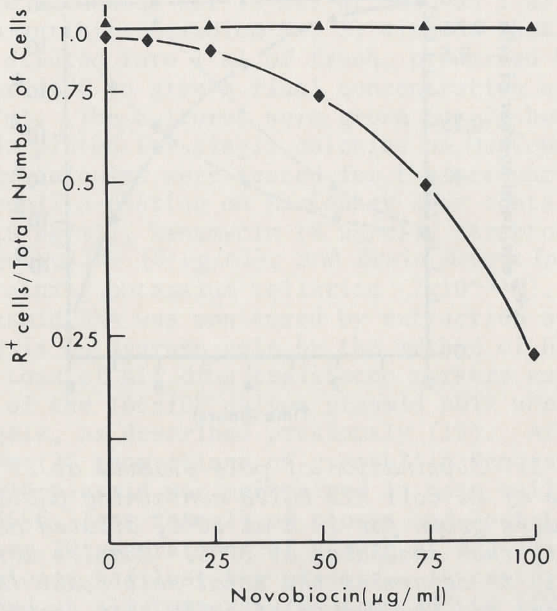

Figure 2. Elimination of pDT4 plasmid by novobiocin at 30°C. Overnight cultures of E. coli *RG192 harboring pDT4 (◆) were diluted in 1 ml Penassay broth to give 5,000 cells/ml, different concentrations of novobiocin were added, and the subcultures were incubated without shaking at 30°C for 18 hours. Cultures were diluted and plated on MacConkey agar and tested for the presence of pDT4 by replica-plating onto antibiotic medium. Control culture contained pSD114 (▲).*

subunit of DNA gyrase.

In the course of studies on the DNA gyrase enzyme, strains of *E. coli* have been isolated which carry a mutation at the *cou* locus and possess a coumermycin and novobiocin resistant DNA gyrase (2). Since pDT4 replication appears to be inhibited preferentially by these antibiotics, we wished to determine if the inhibitor acted directly on the host DNA gyrase or on an enzyme coded by the H plasmid. We therefore transferred pDT4 by conjugation to *E. coli* K12 NI747 cou^{s} $dnaA^{+}$ and NI748 cou^{r} $dnaA^{+}$ (2). The *E. coli* derivatives were tested for elimination of the plasmid at 37°C and at 30°C with various concentrations of novobiocin. Elimination of pDT4 was seen in both strains at 37°C, however, only in strain NI747 was pDT4 eliminated at 30°C after treatment with 100 μg/ml novobiocin. At this concentration about 50% of clones were plasmid-free after overnight growth. The presence of the resistant DNA gyrase enzyme in NI748 protected the plasmid from elimination by novobiocin. No elimination was seen in this host at novobiocin concentrations up to and including 1000 μg/ml.

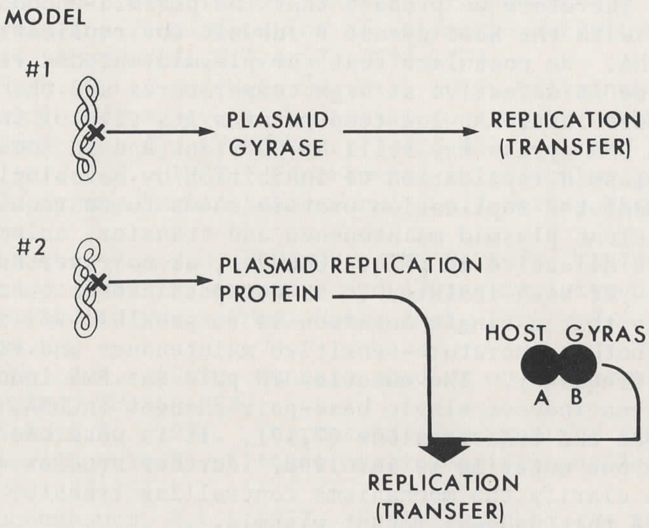

Figure 3. Models to account for behaviour of pDT4 plasmid. The X indicates the mutation in the plasmid.

During our studies with pSD114 and pDT4, we also observed that the transfer frequency of pDT4 was reduced compared with pSD114. Transfer of both plasmids is naturally inhibited at 37°C (1,10,15). At 30°C, pSD114 had a one hundred-fold greater frequency of transfer (5×10^{-4} transconjugants per donor in a 6 hour mating) than the mutant pDT4.

The observation that some plasmids were eliminated by novobiocin whereas others were not led to the suggest that some plasmids might code for their own DNA gyrase which was insensitive to this antibiotic (6). This situation can be visualized as shown in Figure 3, model 1. The behaviour of the mutant pDT4 according to this model might be explained by assuming that the wild-type H plasmid pSD114 encodes its own DNA gyrase which is defective in the mutant. Our evidence that a cou^{R} host DNA gyrase protected pDT4 from elimination by novobiocin indicates that the pDT4 plasmid uses the host DNA gyrase enzyme. Also, the presence of the wild-type H plasmid in host cells does not increase the resistance of the host to novobiocin. Therefore it appears to us that a more plausible model is that shown in Figure 3, model 2. Here the defect in pDT4 lies in another plasmid-encoded enzyme rather than DNA gyrase.

Inhibitors of only the B subunit of gyrase cause pDT4 elimination. Therefore we propose that the plasmid-encoded enzyme interacts with the host gyrase B subunit for replication of plasmid DNA. We postulate that the plasmid-encoded replication enzyme is defective at high temperatures and the plasmid is therefore lost. At low temperatures its site of interaction with DNA gyrase may still be aberrant and in some way predispose plasmid replication to inhibition by novobiocin. The plasmid-mediated replication protein seems to be required for both efficient plasmid maintenance and transfer, as both processes are defective in pDT4. However, as no revertants of pDT4 have yet been isolated it is not possible to conclude absolutely that a single mutation is responsible for the phenotypes of both temperature-sensitive maintenance and reduced transfer frequency. The mutation in pDT4 was EMS induced. This mutagen induces single base-pair changes in DNA, both transitions and transversions (7,17). It is possible that more than one mutation is involved. Further studies are required to clarify the mechanisms controlling transfer and elimination of this unusual mutant plasmid.

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DISCUSSION

Jacoby: Are you observing curing by novobiocin at concentrations that would inhibit an R^- cell?

TAYLOR: A concentration of about 100-150 µg seems to inhibit an R^- cell. We don't see any difference in the level of inhibition by novobiocin of R^+ and R^- cells and the greatest level of elimination is shown at about the concentration that just causes inhibition of cell growth.

Jacoby: Is what grows out chromosomally novobiocin resistant? That is, are you selecting coumermycin-resistant mutants that survive exposure to novobiocin?

TAYLOR: No, I don't think so.

Jacoby: Have you looked?

TAYLOR: We haven't tested the surviving cells specifically.

Jacoby: So possibly there is a special class of resistant mutants which you select with novobiocin that you can't select from a coumermycin-resistant strain, and that is responsible for the mutant.

TAYLOR: Because of the effect of the permeability?

Jacoby: I'm not sure.

TAYLOR: I don't think that they are, but I'd have to test the minimal inhibitory concentration of novobiocin for the surviving cells.

Novick: I might say in connection with that, we have a whole phenomenon parallel to that, which involves our (Staph) penicillinase plasmids. We plate our bacteria on novobiocin and get resistant mutants growing up the next day. About 80% of these have lost their plasmids, and we've never been able to determine what's happening. It's very dramatic: in the act of becoming resistant, they lose the plasmid.

TAYLOR: That's very interesting. We should look for resistance after novobiocin elimination.

Murray: Is it known whether the novobiocin-resistant strain has a resistant gyrase, or is it resistant because of impermeability?

TAYLOR: It's known that it's the DNA gyrase which is resistant. It's not anything to do with permeability.

Smith: Did the 6 hour lag in growth of the cells occur because they grow faster when they lose the plasmid?

TAYLOR: Yes, that's right. When you get the increase in growth rate many of those cells are R^-. It's often found with plasmids which are temperature sensitive that they seem to have some inhibitory effect on growth of the host. For example, Rts1 which is the original temperature-sensitive T group plasmid shows a very similar effect on the host lag phase.

Shapiro: From the graph of the kinetics of loss, is it just that plasmid replication stops and the total number of plasmid molecules stays the same, or if you look at the total number of resistant cells after incubation at high temperature is the plasmid actually lost [degraded]?

TAYLOR: The plasmid is lost, if you're looking at the cell number.

Shapiro: So the number of resistant cells in those populations actually decreases rather than stays constant?

TAYLOR: The number of resistant cells seems to increase for awhile and then remains constant.

CHARACTERISTICS AND INTERACTION WITH BACTERIOPHAGES OF PILI DETERMINED BY A PLASMID OF THE N INCOMPATIBILITY GROUP[1]

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INTRODUCTION

For a number of years it was thought that plasmids of Incompatibility group N (IncN) did not determine pili (5; D.E. Bradley, unpublished). However, a low magnification electron micrograph was eventually published by C.M. To (in reference 1) showing thin filaments on a cell of *Salmonella typhimurium* LT2 carrying the plasmid N3. No experimental or other details were given. Using a different experimental approach to that employed previously (5), large numbers of straight pointed rods have been shown to be determined by N3, so confirming the existence of N pili (D.E. Bradley, *Plasmid*, in press). A survey of all incompatibility groups of the Enterobacteriaceae suggests that there are probably pili for the conjugative plasmids in each. It seems unlikely that there is a pilusless conjugative system.

EXPERIMENTAL METHODS

The non-piliated strains *Escherichia coli* JE2571 and *S. typhimurium* LT2 were used for the detection of pili by electron

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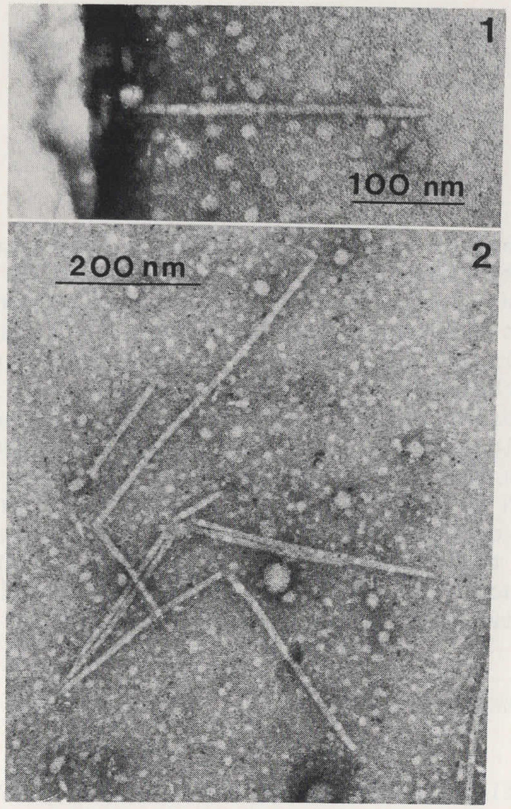
microscopy. Strains carrying the appropriate IncN plasmid (derepressed for transfer) were grown overnight at 37°C on selective plates since previous unsuccessful searches had been confined to bacteria from exponential phase liquid cultures. Cells were mounted for electron microscopy by suspending them with a loop in a drop of 0.1 M ammonium acetate solution, then touching a carbon-coated support grid onto the suspension. Standard negative staining was carried out with sodium phosphotungstate. In order to detect the very small numbers of pili expected to be determined by plasmids naturally repressed for transfer, the same procedure was used save that an extremely thick bacterial suspension was made with most of the cells being washed from the grid before negative staining. Grids were scanned for detached pili rather than those attached to cells. It was thought that this successful procedure produced, in effect, a somewhat concentrated pilus suspension. Larger volumes of N pilus suspension were prepared as described elsewhere (3) using a Thomas Teflon Tissue Grinder to shear the pili from the cells. This was followed by purification using sucrose density gradient ultracentrifugation. The final pilus suspension (in phosphate buffer) contained 500 µg/ml pilus protein.



Figures 1 and 2. N pili synthesized by S. typhimurium *SQ1139(N3) on a cell and from a suspension.*

The observation that IncN plasmids transferred better on solid media than in liquid matings (7) suggested that conjugational behaviour should be studied by some form of plate mating procedure. The following technique (D.E. Bradley, unpublished) was developed to replace the membrane filter mating method, which does not allow the effective introduction of mating inhibitors such as concentrated pilus suspensions or inactivated plasmid-specific bacteriophages. Donor and recipient strains were grown in shake culture and adjusted if required to an optical density of 1.0 (about 2 x 10^9 cells/ml). Equal volumes were mixed, and 0.3 ml of the mixture was spread on a nutrient agar plate and air dried at 37°C. Light microscopy showed that, at the concentrations used, all the bacteria on the plate were in apparent contact with other cells. Islands of cells were in fact formed. After incubation for 1 h, the cells were washed off the plate quantitatively and serial dilutions were spread on selective plates. This method provided an environment which was probably more favourable to mating than the filter method, and had the added advantage of simplicity.

RESULTS

Morphology of N Pili

N pilus rods were not often found attached to cells (Figure 1) when grids were prepared from plate cultures. The frequency of cell-bound rods was extremely variable, even when grids were prepared from the same plate. The highest frequency observed was only 0.8 pili/cell for *S. typhimurium* SQ1139(N3) and 0.4 pili/cell for *E. coli* JE2571(N3). However, detached pili were numerous. The numbers were consistent with a plasmid which was derepressed for pilus synthesis. The ease with which N pili became detached from cells enabled cell-free suspensions to be prepared at high concentration. Electron micrographs of such suspensions (Figure 2) clearly illustrate the short rigid appearance of N pili. Their distal ends are pointed and they appear to be brittle, often breaking into small pieces. They are of variable length (0.1 µm to >1.0 µm) and their thickness is close to that of F pili: about 9.5 nm. The two other IncN plasmids tested, R46 and R199, determine similar pili.

Interaction of N Pili with Bacteriophages

IncN plasmids determine sensitivity to the filamentous bacteriophage IKe (10), whose receptors were originally thought to be located in the outer membrane of sensitive cells (5), perhaps in the form of a very short pilus (8). However, other filamentous phages apparently attach to pilus tips. IncN plasmids also determine sensitivity to lipid-containing phages such as PR4 (11), as do IncW and IncP plasmids. PR4 virions adsorb directly to the tips of both W and P pili (4). These observations taken together with the identification of the N pilus, suggested that the N pilus could be a common receptor for both IKe and PR4. N pilus suspensions were therefore mixed with phage suspensions and studied in the electron microscope after varying periods of incubation. After 20 min incubation with PR4, free pili were observed attached at one end to the virions, probably at the apices of the icosahedral head (Figure 3). Since the free ends of pili complexed with phage were not pointed, PR4 presumably adsorbed at the ends that would have been distal to the cell. In the case of filamentous phage IKe, a long period of incubation was required to demonstrate the expected adsorption to N pili. After incubating a mixture of the phage and an N pilus suspension for 4 h, 20 IKe virions in an observed sample of 50 had become attached to the pointed ends of N pili (Figure 4).

Conjugational Behaviour of the Plasmid N3

Dennison and Baumberg (7) have shown that IncN plasmids can transfer at much higher frequencies on solid media than in liquids. For 30 minute matings they obtained an increase in transfer frequency for R46 from 1.5 x 10^{-5} transconjugants/donor (in liquid) to 8.3 x 10^{-4} transconjugants/donor using a delayed transconjugant selection procedure as follows. Initial plate selection was for recipients only. After incubation, colonies were replica plated onto transconjugant selecting plates. The transfer frequency obtained by the latter method is not as high as would be expected for a plasmid derepressed for transfer. However, using the plate mating technique described above, we have obtained a frequency of about 1 transconjugant/donor (100%) in 1 h isogenic matings. Dennison and Baumberg (7) also found that RP4 (IncP) transferred better by the delayed transconjugant selection method, and we have obtained 100% transfer for the related plasmid RP1 with plate mating.

Concentrated pilus suspensions were incorporated into the plate mating procedure to study their possible effect on mating. Strains JE2571(N3) and JE2571 were mixed in the presence of purified N pili and aliquots were plated on non-

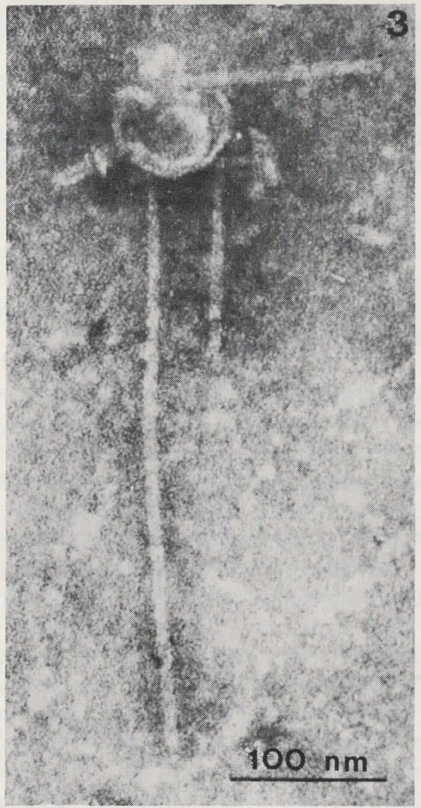


Figure 3. N pili attached to an empty PR4 phage virion (with permission of the Editor of "Plasmid", Academic Press).

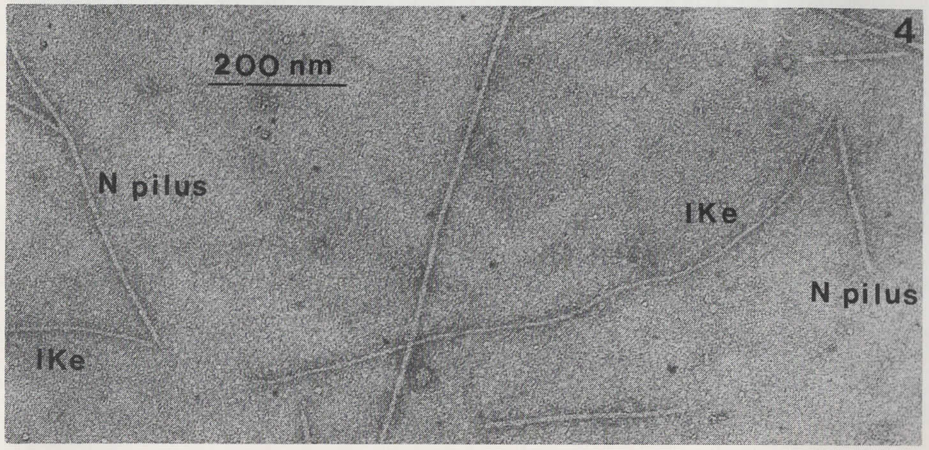


Figure 4. Filamentous phage IKe virions attached to N pili.

selective plates which had been spread with pilus suspension. While the control mating without N pili gave a transfer frequency of 1 transconjugant/donor, mating in the presence of N pili gave $<1 \times 10^{-7}$ transconjugants/donor. Similar results were obtained with RP1 and P pili, indicating with some certainty that N and P pili are involved in conjugation. It is postulated that the large numbers of introduced pili occupied all the receptor sites on the recipient cells, so preventing the pili of the donors from making contact.

Comparison of N Pili with Those Determined by Plasmids of Other Incompatibility Groups

Specific pili have been identified for the groups IncB, IncC, IncJ, IncK, IncM, IncV, and IncX in addition to those already known (D.E. Bradley, in preparation). The remaining groups, IncH1 and IncH2, have not yet been examined. The only pili resembling N pili in morphology are determined by IncM plasmids (M pili), but they do not react with antiserum against N pili. Further, the only other rigid (rod-like) pilus is determined by IncP plasmids (2). However the P pilus is thinner than the N pilus (7.5 nm compared with 9.5 nm) and it has been found that its pilin (pilus protein) has a molecular weight of 13,000 daltons/monomer compared with 13,700 daltons/monomer for N pilin, based on amino acid analysis (T. Chaudhuri, unpublished). Unlike P pili (Figure 5), N pili do not form large aggregates (Figure 2). In spite of these differences, it appears that both types of pilus act as receptors for bacteriophages PR4 and IKe (9).

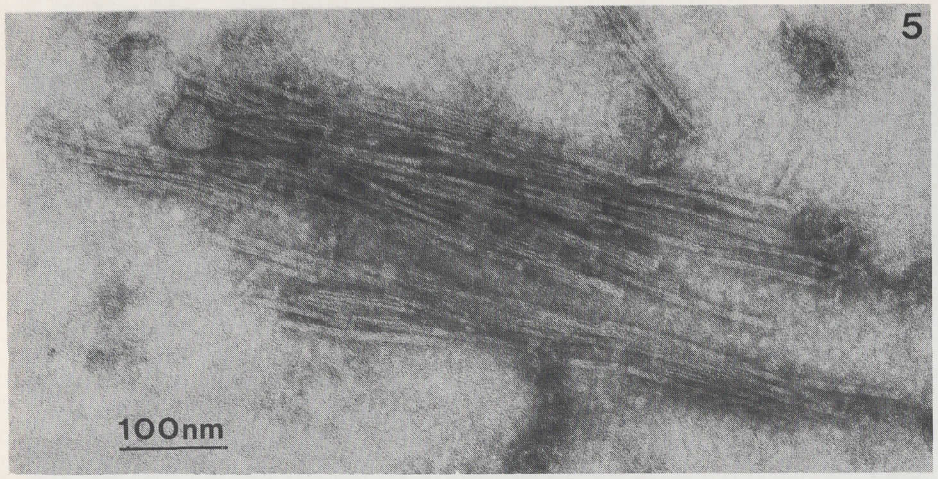


Figure 5. Aggregated P pili from a suspension.

DISCUSSION

These results clearly confirm the existence of N pili. There are probably several reasons why they were not found earlier, not the least significant of which is that searches were carried out on exponential phase broth cultures. The data presented here suggests that N pili are brittle and may well fall off the cells in liquids due to shear forces. This rod-like form of conjugative pilus, which also includes the P pilus, is thus adapted to mating on a solid surface. Flexible organelles such as F pili apparently function equally well in a liquid or on a solid.

It has been clearly demonstrated here that N pilus tips are adsorption sites for phage PR4, as has been shown for P and W pili (4). Coetzee et al. (6) suggest that this kind of apparently non-specific adsorption is due to similarities in the pilus tips, which presumably bind to a common component in the recipient cells. They consider that this, and various other instances, are examples of convergent evolution. The possible existence of such common receptors in recipient cells is being investigated.

ACKNOWLEDGMENTS

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DISCUSSION

Taylor: Has Dr. Bradley looked for pili on cells carrying H1 or H2 plasmids where none have yet been found?

CHAUDHURI: He looked some time ago but not recently. He thinks that such pili might have similar characteristics and might be sheared off the cells and be found in the suspension media. So he is going to look at it again.

MUTANTS OF BACTERIOPHAGE IKe WHICH FORM PLAQUES ON BACTERIA CONTAINING P PLASMIDS[1]

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Previous studies have demonstrated that bacteriphage IKe, once thought to be specific for N plasmids, can propagate in certain *Escherichia coli* strains which carry P plasmids (1). *E. coli* K-12 strain RG172, when carrying any one of several P plasmids, allowed extensive (10^2-10^3 fold) propagation of IKe but no plaque formation. *E. coli* C strain RG176 carrying any P plasmid except R906 did not allow propagation by IKe. Plasmid Rm16b did not promote IKe growth in any strain tested. That the IKe propagation capacity depended on an inefficient receptor coded by the P plasmids (as opposed to the repressed synthesis of a receptor) was shown by the isolation of a host range mutant, IKe*h*1 (previously called IKe*h*), which formed plaques on P$^+$ bacteria. The present study deals with the characterization of IKe*h*1 and three additional host range mutants of IKe; the coincident demonstration that each of the eight P plasmids studied codes for one of five different IKe receptors; and the apparent non-involvement of the IKe receptor of P$^+$ *E. coli* in the conjugation apparatus of these cells, as indicated by the conjugation proficiency of P$^+$ *E. coli* resistant to IKe*h* phage. The P plasmids used in this study are listed in Table 1. The different IKe propagation phenotypes mediated by P plasmids are given in Table 2. The IKe*h* phages were selected by repeated propagation cycles, or direct plaque formation as with IKe*h*2, on various P$^+$ *E. coli* hosts (Table 3). Antigenic relationships of the IKe*h* phages to IKe were confirmed with phage-inhibition tests conducted with 1000 to 10,000

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TABLE 1. *Plasmids of Incompatibility Group P Used in the Present Study*

Plasmid	*Resistance phenotype*[a]	*Original carrier strain*	*Geographical origin*
RP1	*Cb Km Nm Tc*	Pseudomonas aeruginosa	*England*
RP4	*Cb Km Nm Tc*	Pseudomonas aeruginosa	*England*
R26	*Cb Cm Gm Hg Km Nm Sm Su Tc*	Serratia marcescens	*Spain*
R527	*Cb Cm Gm Hg Km Nm Sm Su Tc*	Pseudomonas aeruginosa	*Spain*
R702	*Hg Km Nm Sm Su Tc*	Proteus mirabilis	*U.S.A.*
Rm16b	*Cb Km Nm Tc*	Pseudomonas aeruginosa	*Japan*
R906	*Ap Hg Sm Su*	Bordetella bronchiseptica	*Japan*
RP638	*Cb Km Nm Tc*	Pseudomonas aeruginosa	*S. Africa*

[a] *Resistance abbreviations: Ap, ampicillin; Cb, carbenicillin (and ampicillin); Cm, chloramphenicol; Gm, gentamicin; Hg, mercuric chloride; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Su, sulfa drugs; Tc, tetracycline.*

TABLE 2. *Propagation of IKe by* P^+ E. coli

	IKe propagation factor	
Plasmid[a]	*RG172*	*RG176*
RP1	1.0×10^3	*0.30*
Rm16b	*0.17*	*0.44*
R906	9.9×10^1	1.8×10^2

[a] *Plasmids RP4, R26, R527, R702 and RP638 mediated IKe propagation phenotypes identical to those of RP1.*

TABLE 3. *Derivation of IKe*h *Phages*

Host range mutant phage	*Parent phage*	*Selection procedure*
*IKe*h*1*	*IKe*	*Sequential propagation cycles of phage IKe on RG172(RP4)*
*IKe*h*2*	*IKe*	*Spreading* 10^{11} *pfu*[a] *of IKe directly in lawn of RG172(R906)*
*IKe*h*3*	*IKe*h*1*	*Sequential propagation cycles of phage IKe*h*1 on RG172(Rm16b)*
*IKe*h*4*	*IKe*h*2*	*Sequential propagation cycles of phage IKe*h*2 on RG172(Rm16b)*

[a] *Plaque forming units assayed on an* N_3^+ E. coli *host.*

fold diluted antisera prepared against each of the phages (Table 4). Ten-fold dilutions of these antisera employed in the same test conditions did not inactivate the capacity of phages fd or If to form plaques.

PLASMID HOST RANGE OF IKe*h* PHAGES

Four distinct patterns of host range of the IKe*h* phages have been detected. These patterns correspond to four different kinds of IKe adsorption site coded by the representative plasmids RP1, R702, R906, and Rm16b (Table 5). Host strains carrying selected mutants of RP4, namely pGR10 and pGR11, showed additional diversity of plaque formation.

*TABLE 4. Antigenic Relationships of IKe*h *Phages*

Antiserum prepared against	*Inactivation constant with*				
	IKe	*IKeh1*	*IKeh2*	*IKeh3*	*IKeh4*
IKeh1	$7.9x10^2$	$2.5x10^3$	$3.3x10^2$	- -[a]	- -
IKeh2	$7.2x10^2$	$4.9x10^2$	$1.2x10^3$	- -	- -
IKeh3	$7.9x10^2$	- -	- -	$1.1x10^3$	- -
IKeh4	$5.2x10^2$	- -	- -	- -	$1.2x10^3$

[a] *- - = not determined*

*TABLE 5. Plasmid Host Ranges of IKe*h *Phages*

Plasmid[a]	E. coli *host*[b]	*Plaque formation by phages*				
		PRR1	*IKeh1*	*IKeh2*	*IKeh3*	*IKeh4*
N3	*RG172*	-	+	+	+	+
RP1	*RG172*	+	+	+	+	+
R702	*RG172*	+	+	+	+	+
	RG176	+	+	-	+	+
R906	*RG172*	+	-	+	-	-
Rm16b	*RG172*	+	-	-	+	+
pGR10	*RG172*	-	-	-	+	+
pGR11	*RG172*	+	-	-	+	-

[a] *RM98 mediated the same phage susceptibility phenotype as N3. The plasmids RP4, R26, R527 and RP4 gave phage phenotypes identical to that of RP1. The plasmid pGR10 was a mutant of RP4 selected for the phenotype of resistance to phage PRR1. The plasmid pGR11 was a spontaneous mutant plasmid derived from RP4. All plasmids listed coded for susceptiblity to phage PRD1. All were conjugative.*

[b] *With the exception of R702, all plasmids mediated the same phage susceptibility patterns in the* E. coli *C strain RG176 as they did in the* E. coli *K-12 strain RG172.*

The propagation capacities of IKe*h*1 and IKe*h*2, like their capacities to form plaques, was limited to N$^+$ and P$^+$ bacteria. *E. coli* RG172 and RG176 R$^+$ derivatives were constructed with 29 different plasmids from 18 incompatibility groups other than N and P (1); none could propagate Ike*h*1 or IKe*h*2.

BACTERIAL HOST RANGE OF IKe*h*1 AND IKe*h*2

There was considerable diversity in plaque formation on N3$^+$ and RP1$^+$ bacterial strains from various enterobacterial and non-enterobacterial species (Table 6). In general, the IKe adsorption site specified by the N plasmid is expressed but the IKe*h* site specified by RP1 often is not. Three different *Pseudomonas aeruginosa* strains carrying RP1 did not allow plaque formation by IKe*h*1 or IKe*h*2.

IS THE IKe-IKe*h* ADSORPTION SITE PART OF THE CONJUGATION APPARATUS OF N OR P PLASMIDS?

This question was addressed only by the characterization of conjugation proficiency in mutant strains resistant to phages IKe and IKe*h*. Mutant strains of RG172(RM98) were selected for simultaneous resistance to IKe and the four IKe*h* phages. Ten isolates which maintained the drug resistance

*TABLE 6. Host Range of Phages IKe*h*1 and IKe*h*2 on Various RP1 and N3 Containing Bacteria*

Species (Plasmid)	*Strain number*	*Plaquing of phages*				
		PRD1	*PRR1*	*IKe*	*IKeh1*	*IKeh2*
Escherichia coli *(RP1)*	*RG1901*	+	+	-	+	+
Escherichia coli *(N3)*	*RG1965*	+	-	+	+	+
Salmonella typhimurium *(RP1)*	*GT32*	+	+	-	+	-
Salmonella enteritidis *(RP1)*	*GT57*	+	+	-	-	-
Salmonella enteritidis *(N3)*	*GT55*	+	-	+	+	+
Citrobacter freundii *(RP1)*	*GT36*	+	-	-	+	-
Proteus mirabilis *(RP1)*	*GT47*	-	-	-	-	-
Proteus morganii *(RP1)*	*GT29*	+	-	-	-	-
Proteus morganii *(N3)*	*GT30*	+	-	+	+	+
Yersinia enterocolitica *(RP1)*	*GT60*	+	-	-	-	-
Yersinia enterocolitica *(N3)*	*GT61*	+	-	+	+	+
Yersinia pseudotuberculosis *(RP1)*	*GT23*	+	+	-	-	-
Yersinia pseudotuberculosis *(N3)*	*GT37*	+	-	+	+	+
Achromobacter parvulus *(RP1)*	*GT38*	+	+	-	+	+
Achromobacter parvulus *(N3)*	*GT37*	+	-	+	+	+
Pseudomonas aeruginosa *(RP1)*	*GT63*	+	+	-	-	-
	RG813	+	+	-	-	-
	RG1997	+	+	-	-	-

phenotype specified by the plasmid were purified. All ten had lost sensitivity to phage PRD1 but maintained conjugation proficiency, as assessed by overnight co-cultivation with recipient strains in liquid medium. Similar results were obtained with RG172(RP4): ten isolates selected for resistance to the four IKe phages were resistant to both PRD1 and PRR1 phages and remained conjugationally proficient. With RG176(R906), mutants resistant to IKe 2 gave different results: all of ten mutants resistant to IKe 2 lost their sensitivity to phages PRD1 and PRR1 and their conjugation proficiency. The present evidence indicates that for the P plasmid RP4, and N plasmid RM98, none of the adsorption sites for the phages PRD1, PRR1, or the IKe series is required (in wild type conformation) for the conjugational transfer system of the plasmids.

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DISCUSSION

Willetts: Have you looked in EM to see where the IKe host-range mutants attach: to the cell or to the pilus of P-carrying cells?

GRANT: We have spent a number of weeks attempting to find this out and we have so far lots of nice frames of free phage but that's it; we're still looking.

Lederberg: What are the differences between your two K12 host strains that plated phage IKe?

GRANT: It happens that one of them - RG172 - is a K12 strain and RG176 is a C strain, but we have looked at several K12 strains which behave exactly like RG176 with respect to the phage propagation.

Lederberg: That would explain a lot of the difficulties which some of us have had in plating phage IKe; we may be using the wrong recipient.

GRANT: Well, in the effects we see, the strain differences only pertain to P^+ determinations as far as I know. In other words, N plasmids behave the same in each strain.

Shapiro: Do you know whether the IKe receptor sites are essential for N-mediated conjugation, since that's not essential with RP4?

GRANT: That's a good question. There are conflicting data in the literature. You can select mutants which are resistant to IKe and are still transfer proficient with the N plasmids, but there are some data which indicate that you can also get point mutants which are altered in their transfer proficiency concomitantly with an alteration in their IKe propagation, or plaquing ability. I think the answer is ambiguous at the moment.

CHARACTERIZATION OF THE HOST RANGE OF THE N PLASMIDS[1]

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In the early sixties, Japanese investigators (12) discovered transmissible R factors in shigellae isolates from a dysentery epidemic. Some of those were N plasmids (4). In subsequent years, other investigators (2,5,7,8,9,11) looked at a number of different bacterial species for the presence of R factors transmissible to *Escherichia coli*. They found N plasmids in species of *Proteus*, *Providencia*, *Salmonella*, *Shigella* and *Yersinia*. Besides these reports on the identification of naturally occurring plasmids, the characterization of the host range of the N plasmids has not previously been done.

In the present research, five N plasmids (Table 1), originally isolated from different bacterial species, were used to study the host range. The plasmids were transferred by conjugation into a number of bacterial species. The transfer frequencies were measured and the plasmid stabilities in the new hosts were determined. The matings were done on plates under non-selective conditions. The plasmids were considered to be stable if their hosts still showed all the antibiotic resistance and phage sensitivity plasmid markers after about twenty bacterial generations in a nutrient broth. From these experiments it was found that N plasmids generally transferred at a high frequency and were stable in the *Enterobacteriaceae*. They transferred at a lower frequency or they were not stable in the non-*Enterobacteriaceae*.

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Table 1. N Plasmids Used

Plasmid	*Reference*	*Markers tested*[a]
N_3	13	[b]Sm, Tc
pCF290	this lab	Sm, Cb
RM98	10	Sm, Cb, Tc
R45	6	Cb, Tc
R46	6	Sm, Cb, Tc

[a] *Host sensitivities to phages IKe and PRD1 were tested for all plasmids in addition to the antibiotic resistances used.*

[b] *Abbreviations: Cb, Sm, Tc: resistance to carbenicillin, streptomycin, and tetracycline, respectively.*

The transfer frequencies of the plasmids in the *Enterobacteriaceae* are shown in Table 2. Transfer frequencies to all recipients were usually high with two exceptions. The transfer frequencies to the *Proteus* strains were always low; RP1, on the other hand, transferred to these strains at a frequency of 10^{-1}. *Yersina enterocolitica* also accepted the plasmids at a low frequency; RP1 transferred to *Y. enterocolitica* at a low frequency as well.

When the plasmids were transferred into the *Enterobacteriaceae*, all the markers tested were found in the recipient (except when *Proteus vulgaris* and *P. rettgeri* were the recipients). After their transfer, the plasmids were generally stable in their new hosts. Sometimes there was a slight instability when a few colonies lost one or two plasmid markers. *Enterobacter aerogenes* was unusual because four out of five N plasmids were unstable in this recipient.

Table 3 lists the transfer frequencies of the N plasmids into the non-*Enterobacteriaceae*. The plasmids were generally transferred at a lower frequency, and often some plasmids were not transferred at all. They were also less stable in these recipients. All markers from any one plasmid were rarely detected in the *Pseudomonas* and *Acinetobacter* sp. Only one or two markers were expressed in these recipients.

In general, the transfer frequencies of N plasmids were comparable for a given host. There were, however, some exceptions: *Salmonella typhimurium* LT2, and four non-*Enterobacteriaceae* hosts (*Aeromonas hydrophila*, *A. formicans*, *Achromobacter parvulus*, and *Acinetobacter calcoaceticus* GT16). In these hosts a particular plasmid transferred at a frequency significantly different from that of the other plasmids. For example, R45 had a much lower transfer frequency to *A. hydrophila* than the other N plasmids (Table 4). In the other strains, no single plasmid was consistently anomalous, so no generalizations could be made.

When the plasmids were compared according to their stabilities, RM98 stood out from the other ones, being unstable in the majority of the *Enterobacteriaceae* and non-*Enterobacteriaceae* recipients. All or some of the plasmid markers were lost after growth of the host in a non-selective medium.

From the results of our study, it appears that the host range of the N plasmids is limited. As a control, we used RP1, a P plasmid with a known, wide host range. Its transfer frequency to all the recipients tested was always high ($1.0\text{-}10^{-3}$) with the exception of *Y. enterocolitica* (10^{-6}).

Several possibilities could explain the limited host range: A. The establishment of effective mating between N^+ *E. coli* and bacteria of non-enterobacterial origin might be difficult; some element present on or absent from the outer membrane of either the donor or the recipient cell might prevent the mating. B. Some of the recipient strains could have a restriction enzyme which prevents effective mating. Restriction has been previously reported in matings involving F^+ and F'*gal*$^+$ donors (1). Cornelis and Colson (3) also implicated restriction as the cause for the poor recipient ability of their *Y. enterocolitica* strains. C. Some of the bacterial species might not have an enzyme or attachment site on the inner cytoplasmic membrane necessary for N plasmid replication to occur. This last hypothesis would implicate a relationship in the evolution of bacterial species and their plasmids, the latter (like some viruses) being dependent on host replicative enzymes.

The present data do not permit us to accept or reject any of the above possibilities. Alternative C appears not to be responsible for the low transfer frequencies of the N plasmids into the *Proteus* sp. and *Y. enterocolitica* since the plasmid markers that are transferred into these recipients are stably maintained and can be transferred back into *E. coli* at high frequencies. It is possible that the *Proteus* recipients already contain cryptic N plasmids; however, these would not be typical N plasmids since they do not mediate the plasmid-specific phage sensitivities (IKe, PRD1). All *Proteus* recipients were resistant to these phages; after successful matings, they became sensitive. Although this possibility cannot be excluded, it is perhaps unlikely that a cryptic N plasmid would be present in all the *Proteus* species used. It should be noted that if restriction is involved in the inhibition of N plasmid transfer, it must be by a mechanism to which the P plasmid RP1 is not susceptible (except, possibly, with regard to *Y. enterocolitica*) because the P plasmid was regularly transferred at high frequency.

Table 2. *N Plasmid Transfer Into* Enterobacteriaceae

Species	*Strain designation*	*Frequency of transfer/recipient cell*[a]	*Stability*[b] *(20 colonies tested)*
Escherichia coli	*RG484*	10^{-1}-10^{-2}	*All plasmids are stable*
Yersinia pseudotuberculosis	*GT8*	10^{-1}-10^{-2}	*R46: 20/20 lost Sm*
Yersinia enterocolitica	*AS2426*	10^{-6}-$<10^{-8}$[c]	*RM98: 20/20 lost all markers* *R46: 1/20 lost all markers*
Citrobacter freundii	*GT15*	10^{-1}-10^{-5}	*N_3: 1/20 lost Sm, Tc* *RM98: 1/20 lost Sm*
Enterobacter cloacae	*GT10*	10^{-1}-10^{-4}	*pCF290: 16/20 lost Cb, Sm* *R45: 19/20 lost PRD1s*
Enterobacter aerogenes	*AS1999*	10^{-1}-10^{-3}	*pCF290: 7/20 lost Cb, Sm* *RM98: 12/20 lost all markers* *R45: 15/20 lost Tc, Cb* *R46: 7/20 lost PRD1s*
Proteus vulgaris	*AS1892*	10^{-7}-$<10^{-8}$ *no transfer of R45*	*All transferred plasmids are stable*
Proteus mirabilis	*AS1906*	10^{-7}- 10^{-8} *no transfer of R45*	*All transferred plasmids are stable*
Proteus rettgeri	*AS2371*	10^{-6}-$<10^{-8}$ *no transfer of R45*	*All transferred plasmids are stable*
Salmonella typhimurium	*LT2*	10^{-1}-10^{-7}	*R45: 1/20 lost Tc, IKe^s, PRD1^s*
Salmonella typhimurium	*RG144*	10^{-5}-$<10^{-8}$	*N_3: 4/20 lost Tc* *RM98: 1/20 lost Cb, Sm* *R45: 4/20 lost Cb, Tc*
Salmonella enteritidis	*AS1417*	10^{-1}-10^{-2}	*pCF290: 20/20 lost PRD1^s* *RM98: 3/20 lost Tc*

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[a] *The matings were done by spreading 0.1 ml of an undiluted overnight donor culture over 0.1 ml of a diluted overnight recipient culture on a nutrient agar plate. After incubation at 37°C for 20 hours each plate was replicated to media selective for transconjugants. The frequency of transfer was calculated as the number of transconjugants/number of recipient cells per plate.*

[b] *Markers refer to those listed in Table 1.*

[c] *$<10^{-8}$ means that transfer was not detected.*

Table 3. N Plasmid Transfer Into Non-Enterobacteriaceae

Species	Strain designation	Frequency of transfer/recipient cell[a]	Stability[b] (20 colonies tested)
Achromobacter parvulus	GT2	10^{-2}-10^{-7}	N_3: 3/20 lost IKe^s PRD1^s pCF290: 2/20 lost IKe^s PRD1^s RM98: 1/20 lost IKe^s PRD1^s
Alcaligenes faecalis	B-2710	10^{-3}- 10^{-8} no transfer of pCF290	RM98: 20/20 lost IKe^s PRD1^s R45: 17/20 lost Cb IKe^s PRD1^s
Alcaligenes faecalis	B-2709	10^{-4}- 10^{-8} no transfer of pCF290	RM98: 7/20 lost Cb IKe^s PRD1^s R46: 2/20 lost IKe^s PRD1^s R45: 20/20 lost Cb IKe^s PRD1^s
Aeromonas formicans	AS2297	10^{-1}-10^{-7}	RM98: 20/20 lost Sm Tc R45: 20/20 lost IKe^s R46: 19/20 lost all markers
Aeromonas hydrophila	AS2351	10^{-1}- 10^{-8}	N_3: 3/20 lost Tc Sm PRD1^s 19/20 lost Tc pCF290: 5/20 lost Sm PRD1^s RM98: 19/20 lost Tc R45: 2/20 lost Tc R46: 19/20 lost Tc
Pseudomonas putida	GT111	10^{-7}- 10^{-8} (R45 not tested)	RM98: 5/20 lost Sm
Pseudomonas aeruginosa	GT112	10^{-7}- 10^{-8} no transfer of R45	All the plasmids transferred were stable
Pseudomonas aeruginosa	DT82	10^{-7}- 10^{-8} no transfer of R45	RM98: 20/20 lost Cb Sm
Pseudomonas aeruginosa	RG1998	10^{-6}- 10^{-8} no transfer of R46 (N_3 not tested)	RM98: 20/20 lost Cb Sm
Acinetobacter calcoaceticus	GT16	10^{-1}- 10^{-8} no transfer of RM98, R45, R46 (N_3 not tested)	pCF290: 20/20 lost Cb
Acinetobacter calcoaceticus	AS2282	10^{-7}- 10^{-8} no transfer of N_3	RM98: 20/20 lost Cb R45: 18/20 lost IKe^s PRD1^s R46: 20/20 lost Tc Cb
Acinetobacter calcoaceticus	AS166	10^{-7}- 10^{-8} no transfer of pCF290, RM98	R45: 1/20 lost Tc

[a] As in Table 2.

[b] Markers refer to those listed in Table 1.

Table 4. Frequencies of Transfer to Aeromonas hydrophila *AS2351*

Plasmid	*Frequency/recipient cell*
N_3	7.2×10^{-3}
pCF290	4.3×10^{-1}
RM98	6.1×10^{-2}
R45	$<10^{-7}$
R46	2.2×10^{-4}

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DISCUSSION

Willetts: It wasn't clear to me whether you were losing the whole plasmid or some of the markers, or if you have any experiments to show that even if you've lost antibiotic resistances, you've lost the entire plasmid.

TARDIF: No, sometimes I lost only part of the plasmid, like one or two antibiotic markers when I still had the phage sensitivities. In other instances I had loss of all of the plasmid markers tested. It happened at a low frequency. As I mentioned, in the *Enterobacteriaceae* generally all markers transferred in. Only a few markers were (later) lost but at a low frequency, depending on the plasmid: RM98 lost almost everything, but generally it was only one or two markers.

Willetts: So what you see is a combination of instability of the plasmid's structure as well as actual elimination of the plasmid?

TARDIF: For RM98, I think so.

Novick: In connection with those comments I think that in general it would be nice when one deals with data like this to distinguish structural instability and hereditary instability since they are usually different.

Shapiro: When you have a *Pseudomonas* strain and you see the transfer of only one marker, have you looked at those clones to see whether, when you transfer those plasmids back to *E. coli*, you can recover all the markers?

TARDIF: The retransfer from *Pseudomonas aeruginosa* to *E. coli* doesn't happen, but I had one plasmid which did do that with *Pseudomonas putida*. It has one marker in *Pseudomonas putida* and I recovered two in *E. coli*. It lost one during the process.

Shapiro: One marker wasn't expressed?

TARDIF: Yes, one marker was repressed in *Pseudomonas putida* and was recovered when it was transferred back into *E. coli*. It happened only once.

Table 4. Frequencies of Transfer to Aeromonas hydrophila *AS2351*

Plasmid	*Frequency/recipient cell*
N_3	7.2×10^{-3}
pCF290	4.3×10^{-1}
RM98	6.1×10^{-2}
R45	$<10^{-7}$
R46	2.2×10^{-4}

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DISCUSSION

Willetts: It wasn't clear to me whether you were losing the whole plasmid or some of the markers, or if you have any experiments to show that even if you've lost antibiotic resistances, you've lost the entire plasmid.

TARDIF: No, sometimes I lost only part of the plasmid, like one or two antibiotic markers when I still had the phage sensitivities. In other instances I had loss of all of the plasmid markers tested. It happened at a low frequency. As I mentioned, in the *Enterobacteriaceae* generally all markers transferred in. Only a few markers were (later) lost but at a low frequency, depending on the plasmid: RM98 lost almost everything, but generally it was only one or two markers.

Willetts: So what you see is a combination of instability of the plasmid's structure as well as actual elimination of the plasmid?

TARDIF: For RM98, I think so.

Novick: In connection with those comments I think that in general it would be nice when one deals with data like this to distinguish structural instability and hereditary instability since they are usually different.

Shapiro: When you have a *Pseudomonas* strain and you see the transfer of only one marker, have you looked at those clones to see whether, when you transfer those plasmids back to *E. coli*, you can recover all the markers?

TARDIF: The retransfer from *Pseudomonas aeruginosa* to *E. coli* doesn't happen, but I had one plasmid which did do that with *Pseudomonas putida*. It has one marker in *Pseudomonas putida* and I recovered two in *E. coli*. It lost one during the process.

Shapiro: One marker wasn't expressed?

TARDIF: Yes, one marker was repressed in *Pseudomonas putida* and was recovered when it was transferred back into *E. coli*. It happened only once.

GENETIC AND MOLECULAR SEGREGATION OF PLASMIDS FROM STRAINS CARRYING THE CONJUGATIVE N PLASMID RM98[1]

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V.N. Iyer

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Four conjugation systems are presently recognized among the *Enterobacteriaceae* (7). They are defined by means of bacteriophages specific for host cells in which each conjugation system is expressed. According to this criterion plasmid RM98 is in the N conjugation group, and was the plasmid that was initially used for the isolation of the N specific bacteriophage IKe (4). This phage has since been used to identify cells carrying other members of the N group of plasmids. Useful diagnostic features of the N conjugation system (1,2) include: (i) sensitivity of N^+ cells to phage IKe; (ii) sensitivity of N^+ cells to bacteriophage PRD1; this property is also shared by plasmids of the P and W incompatibility groups; (iii) relatively much higher mating efficiency on solid surfaces than in liquid media.

We have observed that under some conditions the phenotypes specified by this plasmid are unstable. To understand the basis of this instability, we have begun to study the genetic and physical properties of this plasmid. Preliminary evidence is presented which suggests that the $RM98^+$ strain carries interacting plasmids specifying two different conjugation systems only one of each is of the N type.

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I. EVIDENCE FOR CONJUGATIONAL SEGREGATION

Strains carrying the plasmid RM98 have the phenotypes of resistance to ampicillin (Ap^r), spectinomycin, streptomycin and tetracycline (Tc^r); conjugal transfer proficiency (Tra^+); and sensitivity to the phage IKe (IKe^s). For simplicity and also because these were the most frequent classes of segregants, only Ap^r and Tc^r were considered. Figure 1 shows diagramatically the types of conjugational segregants that arose from RM98. Plasmid pMK4 was a spontaneous mutant of RM98 with properties similar to RM98 except in its segregation pattern. The results of some typical experiments are shown in Table 1. Similar data were obtained with the two plasmids in several different donor and recipient strains including recombination-deficient (*recA*) strains. With the parental plasmid RM98 segregation occurred at a high frequency and more than one phenotype was simultaneously missing in different transconjugants. With the mutant plasmid pMK4 the frequency of segregation was relatively much lower and only one type of segregant was observed. Since RM98 and pMK4 were otherwise indistinguishable, the most reasonable hypothesis to explain this observation was that increased stability arose through some structural rearrangement in the plasmid. A further feature of the segregation was that segregants arising from RM98 cells and typified by cells carrying pMK2 were resistant to phage IKe but carried plasmids that were transfer proficient. No such segregant was observed from cells carrying pMK4. Thus the hypothetical

Table 1. Segregation Patterns of RM98 or pMK4 following Conjugation

Plasmid		Frequency of transconjugants per 100 donor cells		Frequencies of different phenotypes of transconjugants (%)					
				Ap^r			Tc^r		
Designation	*Phenotype conferred*^{a}	Ap^r	Tc^r	Tc^s	IKe^r	Tra^-	Ap^s	IKe^r	Tra^-
RM98	$Ap^rTc^rIKe^s$ Tra^+	$2x10^{-3}$	$4x10^{-5}$	97	*nil*	*nil*	90	90	*nil*
pMK4	$Ap^rTc^rIKe^s$ Tra^+	$2x10^{-3}$	$2x10^{-3}$	2	*nil*	*nil*	*nil*	*nil*	*nil*

[a] *Abbreviations:* $Ap^{r/s}$, *resistance or sensitivity to ampicillin;* $Tc^{r/s}$, *resistance or sensitivity to tetracycline;* $Tra^{+/-}$, *conjugal transfer proficiency or deficiency;* $IKe^{s/r}$, *sensitivity or resistance to bacteriophage IKe.*

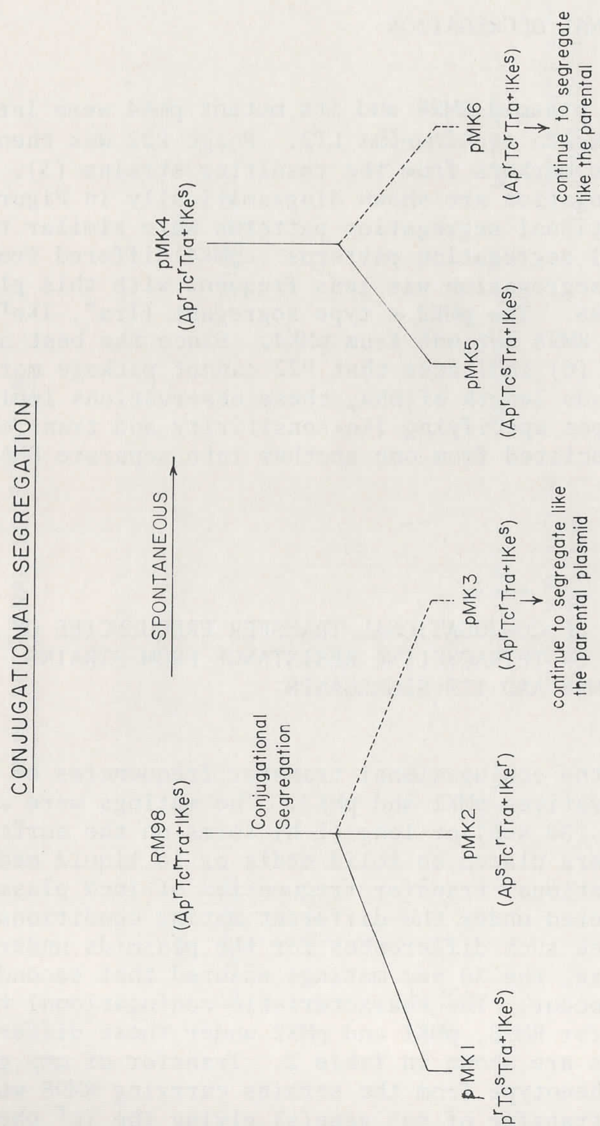
CONJUGATIONAL SEGREGATION
SPONTANEOUS
RM98 ($Ap^rTc^rTra^+IKe^s$)
pMK4 ($Ap^rTc^rTra^+IKe^s$)
Conjugational Segregation
pMK1 ($Ap^rTc^sTra^+IKe^s$)
pMK2 ($Ap^sTc^rTra^+IKe^r$)
pMK3 ($Ap^rTc^rTra^+IKe^s$)
continue to segregate like the parental plasmid
pMK5 ($Ap^rTc^sTra^+IKe^s$)
pMK6 ($Ap^rTc^rTra^+IKe^s$)
continue to segregate like the parental plasmid

Figure 1.

spontaneous rearrangement associated with the change from RM98 to pMK4 apparently resulted in both an increased stability of the plasmid and the absence of a specific class of segregant.

II. TRANSDUCTIONAL SEGREGATION

The parental plasmid RM98 and its mutant pMK4 were introduced into *Salmonella typhimurium* LT2. Phage P22 was then used to transduce markers from the resulting strains (3). The patterns of segregation are shown diagramatically in Figure 2.

The transductional segregation patterns were similar to the conjugational segregation patterns. pMK4 differed from RM98 insofar as segregation was less frequent with this plasmid than with RM98. The pMK2 - type segregant (Tra$^+$, IKe^r) again arose from RM98 but not from pMK4. Since the best available information (6) indicates that P22 cannot package more than one continuous length of DNA, these observations imply that the phenotypes specifying IKe-sensitivity and transfer proficiency dissociated from one another into separate DNA molecules.

III. COMPARISON OF CONJUGATIONAL TRANSFER FREQUENCIES OF AMPICILLIN OR TETRACYCLINE RESISTANCE FROM STRAINS CARRYING RM98 AND ITS SEGREGANTS

We compared the conjugational transfer frequencies of RM98 and its two derivatives pMK1 and pMK2. The matings were conducted for short (30 sec) or long (1 h) terms on the surface of membrane filters placed on solid media or in liquid media. Since the conjugational transfer frequencies of IncN plasmids are markedly altered under the different mating conditions (2), we expected to see such differences for the plasmids under test. Furthermore, the 30 sec matings ensured that secondary matings did not occur. The characteristic conjugational transfer frequencies for RM98, pMK1 and pMK2 under these different mating conditions are shown in Table 2. Transfer of *amp* gene(s) giving the Ap^r phenotype from the strains carrying RM98 was higher than the transfer of *tet* gene(s) giving the Tc^r phenotype. This was observed in short as well as long term matings. In liquid media the frequencies of transfer of both markers in one hour was lower than in one hour matings on the solid surface. However, the frequency of *amp* gene transfer was reduced to a greater extent than the frequency of *tet* gene transfer.

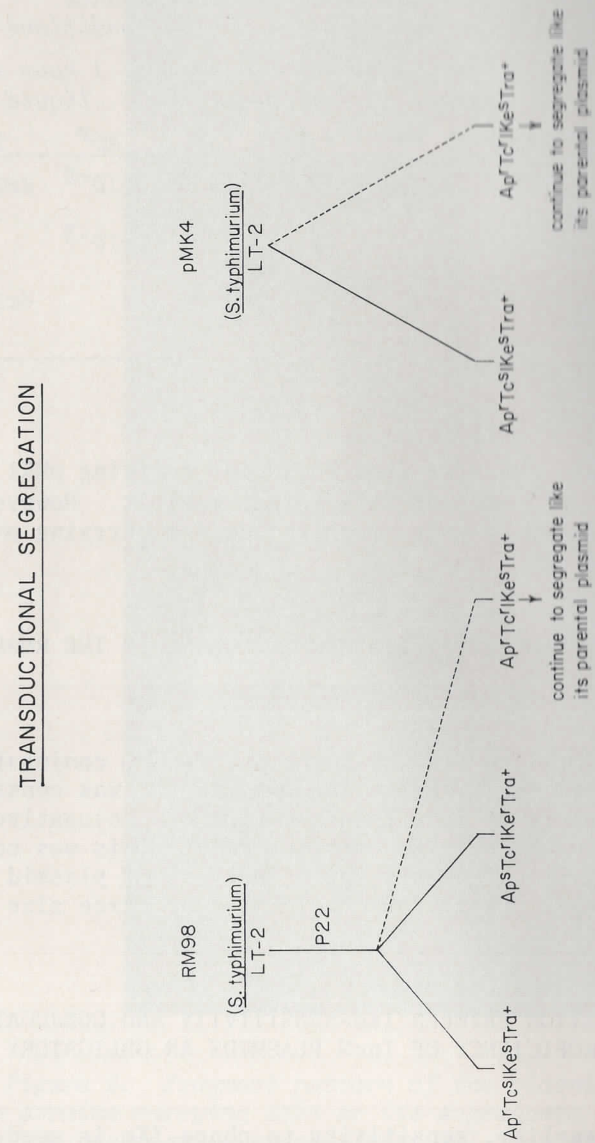
TRANSDUCTIONAL SEGREGATION
RM98
(S. typhimurium)
LT-2
P22
Ap^{r}Tc^{s}IKe^{s}Tra$^+$
Ap^{s}Tc^{r}IKe^{r}Tra$^+$
Ap^{r}Tc^{r}IKe^{s}Tra$^+$
continue to segregate like its parental plasmid
pMK4
(S. typhimurium)
LT-2
Ap^{r}Tc^{s}IKe^{s}Tra$^+$
Ap^{r}Tc^{r}IKe^{s}Tra$^+$
continue to segregate like its parental plasmid

Figure 2.

Table 2. Comparison of Conjugational Transfer Frequencies of Genes Conferring Resistance to Ap and Tc from the Strains Carrying RM98 or its Segregants

Donor Plasmid		Frequency of transconjugants[a] under different mating conditions					
		30 sec on membrane		1 hour on membrane		1 hour in liquid	
Designation	Phenotype conferred	Ap^r	Tc^r	Ap^r	Tc^r	Ap^r	Tc^r
RM98	Ap^r Tc^r IKe^s Tra^+	$2x10^{-3}$	$4x10^{-5}$	40	$2x10^{-1}$	$3x10^{-2}$	$4x10^{-2}$
pMK1	Ap^r Tc^s IKe^s Tra^+	$2x10^{-2}$		70		$2x10^{-2}$	
pMK2	Ap^s Tc^r IKe^r Tra^+		$4x10^{-5}$		$1x10^{-1}$		$4x10^{-2}$

[a] Per 100 donor cells.

The frequency of *amp* transfer from the donor carrying pMK1 was slightly higher than from RM98 in membrane matings. However, the frequencies of *tet* transfer from the donors carrying pMK2 or RM98 were identical (Table 2).

IV. CONFIRMATION OF MULTIPLE PLASMID MOLECULES IN THE RM98$^+$ STRAIN

The evidence obtained from both the short-term conjugation experiments and from the transduction experiments was consistent with the presence of more than one type of conjugative plasmid molecule in the strains carrying RM98. This was confirmed directly by electronmicroscopic analysis of plasmid DNA from this strain. Plasmid DNA molecules of three size classes (26, 52 and 79 megadaltons) were observed.

V. IS THE ASSOCIATION BETWEEN IKe-SENSITIVITY AND CONJUGATIONAL TRANSFER PROFICIENCY OF IncN PLASMIDS AN OBLIGATORY ONE?

As indicated earlier, sensitivity to phage IKe is used to diagnose the presence of an IncN conjugative plasmid. The derivation and properties of pMK2 poses a problem. The plasmid originated in cells that had segregated from a strain carrying RM98, and it could be transferred by conjugation from

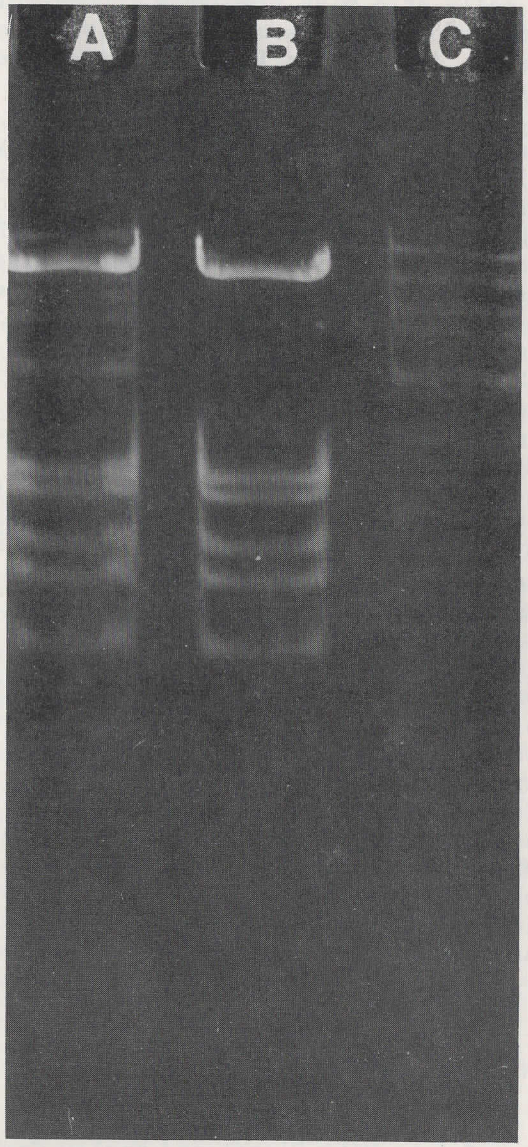

Figure 3. Fragment pattern of the plasmid DNA extracted from strains carrying RM98 or its segregants after digestion with the restriction endonuclease Bgl II. Slot A: RM98; Slot B: pMK1; Slot C: pMK2.

the segregant cells at a high frequency (comparable to the frequency of transfer of the same markers from the RM98$^+$ strain). However, its host cells were insensitive to IKe as well as to all other donor-specific phages. This observation was confirmed with the parental segregant as well as with other secondary strains that have received pMK2 by conjugation. It was inconsistent with previous observations that phage IKe was specific for cells carrying IncN plasmids, and with observations on other conjugational transfer systems where loss of sensitivity to a donor-specific phage was correlated with reduction or loss of transfer ability. We therefore considered a second alternative: that the RM98$^+$ strain carried plasmids which specified two different conjugation systems. One of these was an N system and was associated with sensitivity to phage IKe; the behaviour of plasmid pMK1 was consistent with the presence of such a system. The second conjugative system was specified by plasmid pMK2 (IKe^r Tra$^+$). Since bacteria carrying this plasmid were insensitive to all of the known donor-specific phages (If1, PRR1, R17, IKeh), pMK2 may represent a system that is novel among the *Enterobacteriaceae*.

So far, in the conjugative plasmids that have been studied genetically and physically (1,5) the transfer region occupies a substantial part of the plasmid genome. One might therefore expect that if two plasmids specify different conjugation systems, they would show less similarity to one another in their DNA sequences than if their conjugation systems were related. Figure 3 shows the distinctly different fragment patterns of plasmid DNA extracted from strains carrying RM98, pMK1 and pMK2 after digestion with the restriction endonuclease BglII. These observations support the second alternative. The behaviour of pMK4 could be explained by retention of the IncN system and loss of the second system with the rescue of *tet*. Further studies are in progress to test the validity of these hypotheses and to gain an understanding of the mechanisms by which the various segregants arise from the parental plasmids.

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PLASMID PHAGE P7 CODES FOR A *dnaB* ANALOG[1]

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When bacteriophage P7, also known as ϕamp (7), lysogenizes a cell it confers resistance to ampicillin on that host. In the prophage state it is maintained as a low copy number plasmid (4,9). In this respect it resembles P1, a phage that has a different origin (1). Recent studies have shown that P1 and P7 share considerable genetic and physical homology (2,4,8,9, 12). Evidence has also been obtained to indicate that both P1 and some conjugative R plasmids specify a function similar to *dnaB*, an *E. coli* function essential for DNA replication, and it has seemed possible that this property is widespread among low copy number plasmids in nature (3,5,10,11, Selvaraj and Iyer, unpublished observations). These observations motivated the present experiments which lead to the conclusion that P7 also specifies a *dnaB*-like function.

I. DETECTION OF *dnaB* SUPPRESSING ACTIVITY OF P7

The procedure used to detect the ability of P7 to suppress the temperature-sensitivity of strains carrying a *dnaB(ts)* mutation was based on the work of Ogawa (5) and D'Ari et al. (3) on phage P1, and that of Wang and Iyer (10) on R plasmids. Strain WD266II bearing *dnaB(ts)* was lysogenized with P7 by

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selection for resistance to ampicillin at 30°C. The ability of the resulting lysogen (strain GS200) to form colonies on nutrient agar plates at 37°C was measured. The results given in Table 1 indicate that the P7 lysogens of strain WD266II showed a five-fold increase in the colony forming ability at this restrictive temperature. One temperature-insensitive colony from each of eight independent platings of the lysogen was purified and the prophages in these eight colonies was induced with UV light. The ability of the resulting phages to suppress the temperature-sensitivity was measured as described above. As prophages, seven of the eight phage isolates suppressed the temperature-sensitivity of two different strains carrying a *dnaB(ts)* mutation (strains WD266II and Q1502). The relative frequency of colony formation at 37°C compared with 30°C was about 10^{-1} per cell. This frequency is about 10^{6}-fold greater than that of strain GS200. The similarity between this observation and earlier ones on P1 (5, 3) suggested that one plausible explanation for the increased heat resistance of strains bearing the *dnaB(ts)* mutation was a change in a gene controlling the ability of P7 to suppress *dnaB*. The mutation was provisionally designated *csb* and the hypothetical structural gene for suppressing ability was called *sdb*. *csb* and *sdb* are formally analogous to the *bac* and *ban* genes of P1 (3). *csb5* is a particular mutation of *csb*.

Table 1. Relative Colony Forming Ability of a Strain Carrying a dnaB(ts) *mutation and prophage P7*

Strain[a]	*Allele of the* dnaB *gene in the strain*	*Prophage present*	*Relative Numbers of cfu at 37°C*[b]
WD266II	dnaB266	*none*	*1.0 x* 10^{-8}
GS200	dnaB266	*P7*	*5.0 x* 10^{-8}

---

[a] *The strain GS200 is a P7 lysogen of strain WD266II.*

[b] *0.1 ml of a late exponential phase culture grown at 30°C in Nutrient Broth was spread on each of several Nutrient Agar plates, some of which were then incubated at 30°C and others at 37°C for 2 days before counting the number of colonies. The number of colony forming units (cfu) per ml of the culture was extrapolated from plate counts at 37°C and was divided by the number of cfu per ml calculated from counts at 30°C to obtain these values.*

II. SUPPRESSION OF THE TEMPERATURE-SENSITIVITY OF STRAINS CARRYING DIFFERENT *dnaB(ts)* MUTATIONS BY PHAGE *P7csb5*

We constructed P7 and P7*csb5* lysogens of an isogenic set of strains, members of which differed in their *dnaB(ts)* mutations. The relative colony forming ability of the resulting lysogens was measured at 30°C and 37°C. The results in Table 2 show that the wild-type phage P7 did not suppress the temperature-sensitive phenotype of any of the strains bearing *dnaB (ts)* while P7*csb*5 did. Neither of the two prophages had any effect on the strain carrying the wild-type *dnaB* gene. These results indicated that suppression of the temperature-sensitivity of strains bearing *dnaB(ts)* was not allele specific. DNA synthesis in one of the sets of strains was measured at 30°C and 37°C. The results shown in Table 3 indicate that the inability of a strain bearing *dnaB(ts)* to synthesize DNA at the non-permissive temperature is specifically corrected by the prophage of P7.

Table 2. Suppression of the Thermosensitivity of Different dnaB(ts) *alleles by the prophage* P7csb5

Strain[a]	dnaB *allele*	*Prophage present*	*Relative numbers of cfu at 37°C*[b]
Q125	*wild type*	*none*	*1*
GS401	*wild type*	*P7*	*1*
GS402	*wild type*	P7csb5	*1*
Q1502	dnaB8	*none*	*2.0 x* 10^{-7}
GS403	dnaB8	*P7*	*1.0 x* 10^{-6}
GS404	dnaB8	P7csb5	*7.0 x* 10^{-1}
Q1506	dnaB43	*none*	*<5.2 x* 10^{-8}
GS405	dnaB43	*P7*	*4.0 x* 10^{-8}
GS406	dnaB43	P7csb5	*1.3 x* 10^{-1}
Q1508	dnaB70	*none*	*4.0 x* 10^{-6}
GS407	dnaB70	*P7*	*6.2 x* 10^{-6}
GS408	dnaB70	P7csb5	*3.0 x* 10^{-1}
Q1610	dnaB266	*none*	*5.0 x* 10^{-8}
GS409	dnaB266	*P7*	*8.4 x* 10^{-7}
GS410	dnaB266	P7csb5	*5.0 x* 10^{-1}

---

[a] *These strains were isogenic except for the* dnaB *allele and the prophage. The "GS" strains were constructed by lysogenizing the corresponding "Q" strains carrying the* dnaB(ts) *mutation or the wild type* dnaB *gene with phages P7 or P7*csb5.

[b] *The colony forming ability was measured as described in Table 1.*

Table 3. Net Increase in the DNA of a Strain Carrying a dnaB(ts) *mutation and P7*csb5 *or P7 after 3 hours at different temperatures*

Strain[a]	dnaB *allele*	*Prophage present*	*Net increase in DNA after 3 hr at the indicated temperature*[b]	
			30°C	*37°C*
Q1506	dnaB43	*none*	*21.8*	*1.1*
GS405	dnaB43	*P7*	*21.9*	*1.4*
GS406	dnaB43	*P7*csb5	*20.0*	*73.0*

[a] *The strains GS405 and GS406 are P7 and P7*csb5 *lysogens of the strain Q1506 respectively.*

[b] *The strains were grown in LB broth (6) containing [methyl-^{3}H] thymine at 30°C to mid-exponential phase. They were then diluted 50-fold into prewarmed medium of the same composition and the acid-insoluble counts per 0.1 ml of the culture were obtained just after the culture was diluted and after 3 hr at the indicated temperature. The counts after 3 hr of incubation of the culture were divided by the counts at time zero to calculate the net increase in DNA.*

III. THE ABILITY OF P7*csb5* TO RESCUE A STRAIN CARRYING THE *dnaB266(am)* MUTATION

The *dnaB266* mutation has been shown to be a conditionally lethal amber mutation (5). The strain Q1550 bearing this mutation has been shown to be viable because it carries phage P1Cm*bac* which codes for a *dnaB* analog (3). Since in the lysogenic state P1 and P7 are incompatible with one another, P7 or P7*csb5* should be able to replace P1Cm*bac* in the strain Q1550 only if it also possesses a similar rescuing ability. Strain Q1550 (*dnaB266(am), sup$^+$*) was lysogenized separately with P7 and P7*csb5* selecting for ampicillin-resistance. The frequency of lysogenization was 2.5 x 10^{-1} and 2.2 x 10^{-2} respectively and in both cases approximately 2% of the ampicillin-resistant (Amp^r) colonies had lost the chloramphenicol-resistance marker associated with P1Cm*bac*. The chloramphenicol-sensitive (Cm^s) colonies thus obtained were tested for the presence of the different prophage immunity systems of P1 and P7. This was done by screening the colonies for sensitivity to phages P1 and P7. Since P1 and P7 are heteroimmune, a P1-lysogen or P7-lysogen can be lytically infected by P7 or P1 respectively, but not by the homologous phage (2,9). The phage-sensitivity studies showed that all of the five independent Cm^s derivatives of the strain Q1550 obtained by infection with P7 had retained the P1-immunity system while five out of nine Cm^s colonies derived from P7*csb5*-infection had lost it. The colonies that had lost the P1-immunity system were considered to have lost the phage P1Cm*bac*, while the Cm^s colonies that retained it were believed to arise by recombination between the two phages i.e., P1Cm*bac* and P7 or P1Cm*bac* and P7*csb5*. The Cm^s colonies that have lost the P1 immunity system are unlikely to be recombinants between P1Cm*bac* and P7*csb5* because the frequency of lysogenization of P7 and P7*csb5* is similar and yet P7-infection (in contrast to P7*csb5*-infection) did not result in the loss of the P1-immunity system. Thus P7*csb5* but not P7 displaces P1Cm*bac* from the strain Q1550. This is interpreted as evidence to support the notion that P7*csb5* codes for a *dnaB* analog.

IV. WILD-TYPE PHAGE P7 CARRIES A *dnaB* ANALOG GENE

Since P1 and P7 are closely related we examined the possibility of constructing a recombinant of the phages P1 and P7 in which the *dnaB* analog gene of P7 would now be associated with the phage P1. Phage P1Cm*bac ban* is a derivative of P1Cm*bac* in which the *ban* gene (the structural gene for the *dnaB* analog) is mutated and consequently the phage has lost its ability to suppress the temperature-sensitivity of *dnaB(ts)*-bearing strains. Moreover, this phage does not rescue a strain carrying the *dnaB266(am)* mutation. However, the *bac* allele which controls the expression of the *ban* gene is not altered (3).

We crossed P1Cm*bac ban* with the wild-type P7 in strain C600 and the resulting phage lysate was selectively enriched for phage particles with the P1-immunity system by growing the phages on a P7 lysogen. Since P7 cannot replicate in this strain while P1 can, the phage lysate obtained after this step would predominantly have P1-type particles. These P1 particles were then assayed for the ability to suppress a *dnaB(ts)*-bearing strain. The results (see Table 4) show that the lysate of neither P1Cm*bac ban* nor that of P7 is able to render the *dnaB(ts)*-bearing strain temperature-resistant while the lysate containing recombinant particles is able to do so. These results support the hypothesis that the wild-type phage P7 does carry a structural gene coding for a *dnaB* analog and that it is constitutively expressed in P7*csb5*. Furthermore it appears that the *sdb* gene of P7 and the *ban* gene of P1 are functionally similar to the extent that the *sdb* gene can be expressed under the control of the *bac* gene of P1.

*Table 4. Phage crosses between P1Cm*bac ban *and P7*[a]

Phage preparation	*Number of temperature-sensitive lysogens of strain WD266II carrying* dnaB266 *at 37°C, per phage pfu*
*P1Cm*bac ban	*1.0 x* 10^{-9}
P7	*4.0 x* 10^{-8}
*P1Cm*bac ban *x P7, regrown on a P7 lysogen*	*2.3 x* 10^{-6}

[a] *Phage crosses were performed by co-infecting* E. coli *strain C600 with the two phages involved. Progeny particles were plated on strain GS401 (strain Q125 lysogenized with phage P7). Since P1 and P7 are heteroimmune the parental and recombinant phages with a P1-immunity system can grow in this strain while the homoimmune phage P7 cannot. The P1 particles thus enriched were harvested. 0.1 ml of this lysate was mixed with 0.1 ml of an exponential phase culture of the strain WD266II. The mixture was spread on a nutrient agar plate and incubated at 37°C for 3 days. The number of lysogens is expressed as the number of colonies formed at 37°C per plaque forming unit (pfu) of the lysate. The lysate was assayed on strain Q125 to determine the titer of the phage pfu.*

In summary, the wild-type phage P7 does not suppress the temperature-sensitivity of *dnaB(ts)*-bearing strains while a mutant of it, P7*csb5* does. Both P7 and P7*csb5* carry *dnaB* analog genes but only P7*csb5* is capable of rescuing a *dnaB(am)*-bearing strain. Therfore the *csb5* allele appears to control the expression of the *dnaB* analog gene (*sdb*) in P7. The *sdb* gene can also be expressed under the control of the *bac* gene of P1. These studies indicate that the *dnaB* analog function of phage P1 and P7 are probably similar.

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DISCUSSION

Shapiro: Danielle Schwartz has studied the expression of the *dnaB* analogue genes extensively in ϕP1 and it appears that mutations like the *csb* are fusions of the B analog gene, which is normally expressed only in lytic growth, to genes which are only expressed as prophage genes. So there's a regulation which turns some genes on in the plasmid phase of the prophage and others which turn genes on in the lytic phase. In her mutants, which I presume are the same as your because they are dominant, the *dnaB* gene is now hooked up to genes which are expressed only when the plasmid is in the prophage state.

SELVARAJ: I see, I was not aware of that. Has it been published yet?

Shapiro: It's been submitted to Molec. Gen. Genetics. [Published in vol. *174:*173-188(1979), ed.]

CONSTRUCTION AND CHARACTERIZATION OF A RECOMBINANT PLASMID CONTAINING THE ENTIRE *BIOTIN* GENES OF *E. coli* K12[1]

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SUMMARY

Biotin genes of *E. coli* K12 were isolated by cleaving *bio* transducing λ*biot*124-10 phage DNA with *Eco*RI restriction enzyme. The *Eco*RI cleaved fragment was joined to *Eco*RI cleaved pMB8 plasmid DNA by polynucleotide ligase. pMB8 is a derivative of ColE1 and contains a single *Eco*RI site. Host cells carrying this plasmid are immune to colicin E1. The pMB8-*bio* plasmid chimaera was used to transform *E. coli* K12 SK80 (*rec*A *bio*Δ61). The bio^+ and ColE1 immune transformants were selected. Clones carrying the pMB8-*bio* plasmids were found to produce approximately 200 copies of plasmid DNA per cell at the end of the log period when grown in a defined (M9) medium without any addition of chloramphenicol. These clones also produced a large amount of biotin and mRNA specified by *biotin* genes. Other biochemical and biological properties of the pMB8-*bio* recombinant plasmid are discussed.

INTRODUCTION

We have previously reported the procedure for isolation

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and characterization of the entire *biotin* (*bioABFCD*) genes of *E. coli* K12 (4). The *bio* gene cluster was obtained after cleaving the *bio* transducing λ*biot*124-10 phage DNA with the restriction enzyme *Eco*RI. The *Eco*RI fragment number 2 which contained the *bio* genes was biologically and biochemically active as shown by its ability to act as a template for *in vitro* transcription and translation (4). We have cloned this *Eco*RI fragment containing the *bio* genes using pMB8, a mini ColE1 plasmid, as the cloning vehicle. The pMB8-*bio* plasmid chimaera was transformed into a Bio^- auxotrophic strain of *E. coli* K12. Cohen et al. (3) have also reported the construction of ColE1-*amp* plasmid containing the entire *bio* gene cluster of *E. coli* using a different λ transucing phage as the source of *bio* DNA. The pMB8-*bio* plasmid chimaera, however, possesses several properties which are very helpful for studies of the regulation of *bio* genes at the molecular level. In this report some of these biological and biochemical properties of the pMB8-*bio* plasmid-chimaera are presented.

CLONING OF *BIOTIN* GENES OF *E. COLI* K12 USING pMB8 AS THE CLONING VEHICLE

Isolation and characterization of the *bio* genes (*bioABFCD*) of *E. coli* K12 was described previously by DasGupta et al. (4). An *Eco*RI cleavage product of λ*biot*124-10 phage DNA (fragment number 2) consisted of approximately 8960 base pairs and contained the entire *bioABFCD* gene cluster of *E. coli* K12 (Figure 1). Its molecular weight of 5.7 Mdal was determined from an agarose electropherogram using *Eco*RI cleaved λDNA fragments as control. DNA comprising the *bioABFCD* genes in this fragment was estimated to be 12.7% of the λ^+ length. In addition to *bio* genes the fragment contained approximately 6% λDNA. The *bio* DNA fragment acted as an efficient template in transcription experiments *in vitro*. The 5'-termini were adenosine in both *bioA* and *bioBFCD* RNA transcripts synthesized *in vivo* and *in vitro* (C.K. DasGupta and A. Guha, unpublished data). In a transformation assay using a Bio^- auxotroph *E. coli* SK61 (8) as the recipient and *Eco*RI fragment number 2 as the transforming DNA, Bio^+ transformants were obtained.

CONSTRUCTION OF pMB8-*BIOTIN* PLASMID CHIMERA

The mini ColE1 plasmid, pMB8, has a single *Eco*RI cleavage site (1). The construction of this plasmid and some of its

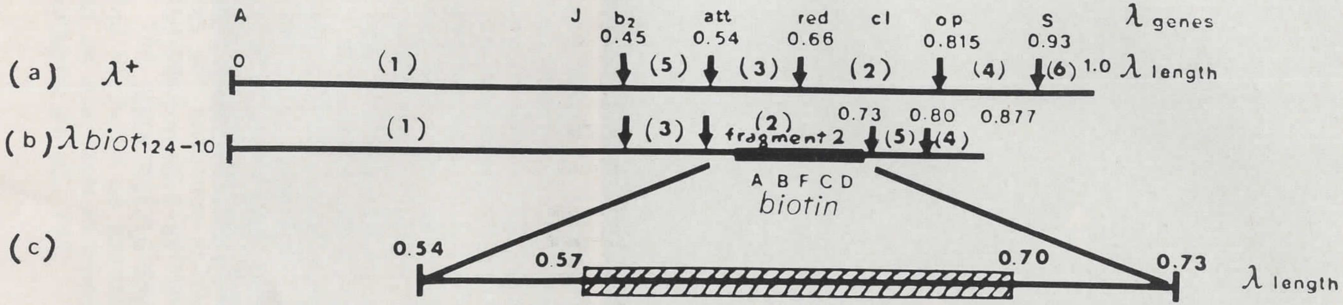
A
J
b2
0.45
att
0.54
red
0.66
cI
op
0.815
S
0.93
λ genes
(a) λ+
0
(1)
(5)
(3)
(2)
(4)
(6)
1.0 λ length
(b) λbiot124-10
(1)
(3)
(2)
fragment 2
0.73
0.80
0.877
(5)
(4)
A B F C D
biotin
(c)
0.54
0.57
0.70
0.73
λ length

Figure 1. Restriction endonuclease Eco*RI maps of (a) λ and (b)* λbiot-*124-10 phage DNAs. The arrows indicate cleavage sites. The numbers in parentheses designate fragments which are in decreasing order of size; a full length of λDNA (1 unit) contains about 48000 base pairs (5).* Eco*RI fragment number 2 represents the cleavage product which contains the entire* bioABFCD *genes.* Eco*RI fragment number 2 is enlarged in (c) to show the segment 0.127 λ length which contains the entire* biotin *genes and 0.3 length of λDNA on either side. The* bio *genes contain approximately 601 kilobase pairs.*

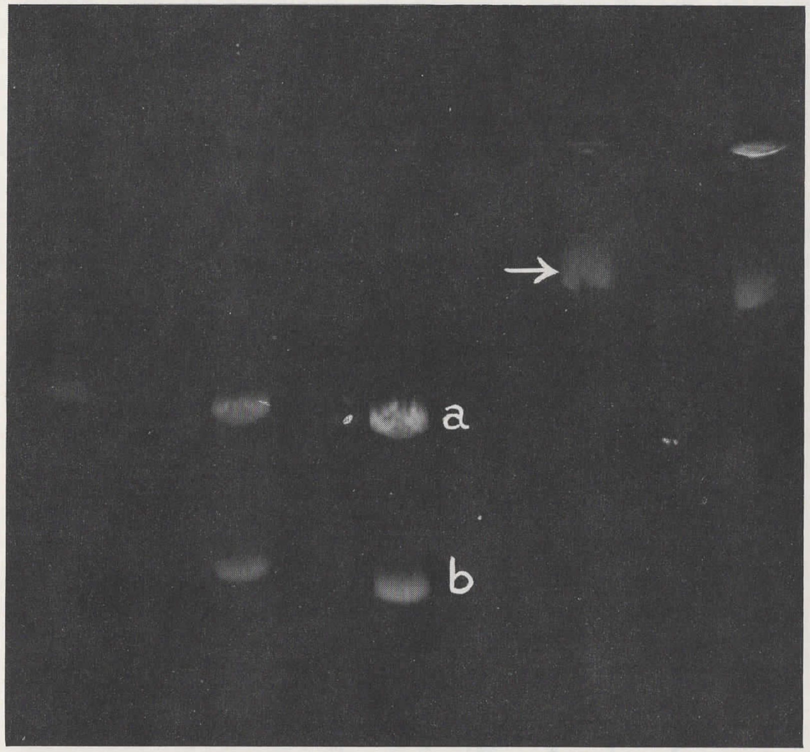

Figure 2. Agarose gel electrophoresis of Eco*RI digests of pMB8-*bio *DNA. DNA was isolated from SK80-303 grown in M9 medium lacking biotin (7) in presence of 200 μg/ml chloramphenicol following the procedure described by Guerry et al. (6). The cell lysate was electrophoresed on 1.2% agarose gel and the gel was stained with ethidium bromide. The DNA band marked by an arrow corresponds to 7.4 Mdal. The DNA was eluted by the technique of DasGupta et al. (4) and was visualized under the electron microscope. The eluted DNA was treated with* Eco*RI restriction endonuclease and the cleavage products were electrophoresed in 1.2% agarose gel. The pMB8-*bio *DNA circles were cleaved into two fragments. The fragment marked 'a', corresponding to 5.7 Mdal, is the* Eco*RI fragment number 2 of λ*bio*t124-10 phage DNA which contains the entire* bio *genes. The lower band marked 'b' is linear pMB8 plasmid DNA molecules with a molecular weight of 1.7 Mdal.*

biological properties were described by Rodriguez et al. (9). The *Eco*RI cleaved pMB8 plasmid DNA and *Eco*RI fragment number 2 of λ*biot*124-10 phage DNA containing the entire *bio* genes were ligated and transformed to a Bio^- auxotroph *E. coli* SK80 which is a *rec*A derivative of SK61 (8), following the technique described by Cohen et al. (2). The transformed cells were selected by their ability to grow on minimal agar plates lacking biotin. The Bio^+ transformants were further selected on agar plates containing colicin E1. One of the finally selected clones, designated as SK80-303, grew on the medium lacking biotin and was also immune to colicin E1. Plasmid DNA was isolated from the transformed cells and rebanded by electrophoresis on agarose gel (Figure 2) after *Eco*RI cleavage. Both the *bio* DNA fragment and the plasmid pMB8 DNA formed bands on gels in their respective original positions. Figure 3 shows an electron micrograph of the pMB8-*bio* plasmid DNA. The estimated length as measured from the circular molecule was 3.8 μ, corresponding to 11.6 kb (1 μ corresponds to 3 kb).

BIOLOGICAL PROPERTIES OF THE TRANSFORMED HOST SK80-303, CARRYING THE PLASMID CHIMERA

Data given in Table 1 show that the chimaeric plasmids in the transformed cells were not very stable. By the time the culture reached the stationary phase approximately 40% of the

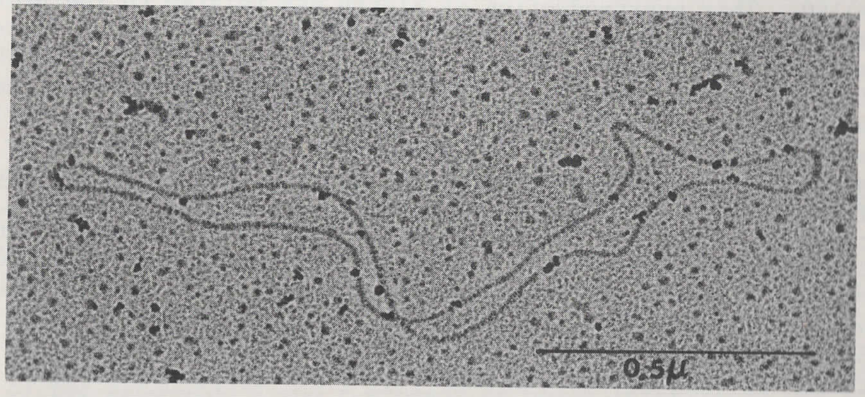

*Figure 3. An electron micrograph of the pMB9-*bio *plasmid DNA. The covalently closed circular DNAs were isolated from strains SK80-303 as described in Figure 2. The DNAs were spread on electron microscope grids following the technique of DasGupta et al. (4).*

cells were cured of the plasmid, as determined by the relative numbers of colonies growing on minimal agar plates with biotin compared with those growing on minimal agar (selecting Bio^+ colonies) or minimal agar containing colicin E1 and biotin (selecting Col $\mathrm{E1}^+$ cells). Those cells which were colicin E1-sensitive ($\mathrm{ColE1}^-$) were also unable to grow on medium without biotin.

The copy number of plasmid DNA per cell in SK80-303 was determined by growing the cells in the presence of ^{3}H-thymidine (25 Ci/ml, New England Nuclear Corp.). After lysis of the cells the lysate was run in agarose gel electrophoresis and the TCA precipitable radioactivities present in the eluted chromosomal and plasmid DNA bands were compared. Mean estimates of the plasmid copy number per chromosome obtained from 10 different experiments indicate that the plasmid-chimera, pMB8-*bio*, produced about 200 copies per chromosome when grown in M9 medium alone and the number was enhanced only 1.6 times (to 310 copies per cell) after chloramphenicol treatment. Therefore strain SK80-303 could overproduce biotin and *bio* gene specific mRNA because a large number of plasmid DNA copies per chromosome were produced even without addition of chloramphenicol.

The production of biotin by strain SK80-303 was examined and compared with the level of biotin excreted by a plasmid free Bio^+ prototroph *E. coli* W3350. *E. coli* T5-2 in which the entire *bio* genes are deleted was used for the bio-assay. Strain Sk80-303 produced 100 fold excess of biotin compared to W3350. This overproduction of biotin was related to the production of large amounts of *bio* specific mRNA. Table 2 shows that in the absence of any exogenous biotin supply 4.24% [^{3}H]-labeled RNA isolated from strain SK80-303 hybridized with the *bio* DNA and the ratio of *bio* ℓ and *r* RNA was found to be 30:70 as for the [^{3}H]-RNA isolated from W3350 which does not carry pMB8-*bio*. The total amount of *bio* specific [^{3}H]-RNA isolated from W3350 in the derepressed condition was 0.18% (line 1, column 7, Table 2). Although 10^{-3} μg/ml biotin was sufficient to completely repress the transcription of both the bio ℓ and *r* RNA (7), a concentration even 50-100 fold above this level was unable to repress the *bio* genes in cells of strain SK80-303 carrying pMB8-*bio*. Since the number of repressor molecules was insufficient to bind to all the operators of the DNA molecules carried by 200 plasmid copies per chromosome, the presence of excess co-repressor (*d*-biotin) in the medium was still unable to repress transcription of *bio*-RNA in strain SK80-303. This strain is now being used for further studies on the regulation of biotin biosynthesis, *bio* mRNA isolation and biotin biosynthetic enzymes.

Table 1. Stability of pMB8-bio *in the Host Strain* E. coli *SK80-303*[a]

Growth period (min)	*Relative number of bacterial colonies per plate*		
	M9 agar + biotin	*M9 agar without biotin*	*complete M9 agar with colicin E1*
0	100	62.6	69.1
120	100	70.8	72.3
240	100	64.2	61.2
600	100	60.7	59.6

[a] *Overnight culture of SK80-303 grown in M9-glucose medium (7) was inoculated to a flask containing fresh M9-glucose medium and was allowed to shake at 30°C. At times 0, 120, 240 and 600 min after the transfer, a 0.01 ml was taken out of the flask and diluted to 10^{-4} in M9 buffer (7). The samples were diluted further in M9 buffer (7) and 0.2 ml from the appropriate dilution was plated on test media. The plates were incubated at 30°C for 48 hrs. and the numbers of colonies were counted. The results shown in the table were the average of 5 different experiments.*

Table 2. Production of bio *Specific ^{3}H-RNA in Strain SK80-303*

Strain	*Input* [^{3}*H*] *RNA (cts/min)*	*^{3}H-RNA annealed with* λbiot*124-10 phage DNA (cts/min)*		*Percentage* bio *RNA hybridized*		*Percentage total* bio *RNA (ℓ+r)*
		ℓ *strand*	r *strand*	ℓ *strand*	r *strand*	
SK80-303	*18.6x10^5*	*1004.4*	*2343.6*	*0.054*	*0.126*	0.18
W3350	*6x10^3*	*76.2*	*178.2*	*1.27*	*2.97*	4.24

[a] *Labeled RNA was isolated by hot phenol extraction (7) after exposure of biotin-starved cells to 20 μCi/ml* [^{3}*H*]*-uridine for 12 min at 37°C. It was then annealed with a large excess of the separated* ℓ *and* r *strands of phage* λbiot*124-10 DNA by the liquid hybridization technique (7).*

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